ENDOCRINE AND MOLECULAR CHANGES ASSOCIATED WITH OVARIAN FOLLICULAR DOMINANCE IN CATTLE

BY

RODOLFO LUZBEL DE LA SOTA

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Dedicated to

Teresita, Tomás, Matías

and

my mother and my late father

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TABLE OF CONTENTS

page

ACKNOWLEDGMENTS iii

LIST OF TABLES viii

LIST OF FIGURES x

LIST OF ABBREVIATIONS xv

ABSTRACT xvii

CHAPTERS
1 INTRODUCTION 1

2 LITERATURE REVIEW 4
  Ovarian Follicular Growth and Development 4
    Ovarian Follicular Growth and Development in Embryonic Life 4
    Gonadotropin Receptors in Ovarian Follicles 6
    Studies of Bovine Follicular Development: Macroscopic and Microscopic Techniques 8
    Studies of Bovine Follicular Development: Ultrasonic Imaging 11
    Follicular Dynamics During the Bovine Estrous Cycle 12
    Experimental Models Utilized to Study Follicular Dynamics During the Bovine Estrous Cycle 17
  Steroidogenic Enzymes and Steroid Hormones 19
    Steroidogenesis in Bovine Nonovulatory and Preovulatory Follicles 19
    Two-Cell, Two-Gonadotropin Theory 22
    Steroids Concentrations in Follicular Fluid: Indices of Dominance and Atresia 24
    Steroidogenic Enzyme Gene Expression in Ovarian Follicles . . 26
    Regulation of Steroid Hormones Production at the Ovarian Level 32
    Regulation of Steroid Hormones Gene Expression at the Ovarian Level 35
  Inhibin/Activin 36
    Characterization of Inhibin Molecules in Follicular Fluid 37

Postranscriptional Processesing of Inhibin Forms in Follicular Fluid 38
Endocrine, Paracrine and Autocrine Roles of Inhibin 41
Radioimmunoassay and Immunoradiometric Assay of Inhibin in Follicular Fluid 43
Characterization of Different Forms of Inhibin in Follicular Fluid: Immunoblot Analysis 44
Inhibin Gene Expression 45

Insulin-like Growth Factors (IGF) and IGF-Binding Proteins (IGFBP) . 48
Characterization of IGF-I and IGF-II Molecules 49
Characterization of IGF-I and IGF-II Receptors 49
Concentrations of IGF-I and IGF-II in Follicular Fluid 50
Biologial effects of IGF-I and IGF-II at the ovarian level 51
IGF-binding Proteins in Follicular Fluid 52
Mechanisms that Regulate the Amounts of IGFBP in Follicular Fluid 54
Ovarian Gene Expression of IGFBP 57

3 PROGESTERONE CONCENTRATION AND mRNA LEVELS FOR P450 CHOLESTEROL SIDE-CHAIN CLEAVAGE AND P450 3β-HYDROXYSTEROID DEHYDROGENASE IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES . . 60

Introduction 60
Material and Methods 65
Results 73
Discussion 92

4 ANDROSTENEDIONE AND 17β-ESTRADIOL CONCENTRATIONS AND mRNA LEVELS FOR P450 17α-HYDROXYLASE-17,20 LYASE AND P450 AROMATASE IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES 104

Introduction 104
Material and Methods 108
Results 111
Discussion 131

5 CONCENTRATIONS OF INHIBIN AND INHIBIN-α SUBUNITS AND mRNA LEVELS OF INHIBIN α- AND β_A SUBUNITS IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES . . 147

Introduction 147
Material and Methods 152
Results 155
Discussion 181

6 INSULIN-LIKE GROWTH FACTOR-I (IGF), IGF-II AND IGF-BINDING PROTEINS (IGFBP) CONCENTRATIONS IN FOLLICULAR FLUID AND IGFBP-2 mRNA LEVEL IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES 201

Introduction 201
Material and Methods 205
Results 211
Discussion 232

7 GENERAL DISCUSSION AND CONCLUSIONS 246

REFERENCES 270

APPENDIX 1 RNA EXTRACTION IN OVARIAN FOLLICLE WALLS 294

APPENDIX 2 NORTHERN BLOTTING 297

APPENDIX 3 DOT BLOTTING 300

APPENDIX 4 PREHYBRIDIZATION AND HYBRIDIZATION 302

APPENDIX 5 MULTIPRIME DNA LABELING (AMERSHAM KIT RPN.1601) 304

APPENDIX 6 POLY-A RNA ISOLATION 305

APPENDIX 7 IGF-I AND IGF-II RIA IN FOLLICULAR FLUID 307

APPENDIX 8 LIGAND BLOTTING FOR IGF-BINDING PROTEINS 312

APPENDIX 9 IMMUNOPRECIPITATION OF IGF-BINDING PROTEINS . 317

BIOGRAPHICAL SKETCH 319

LIST OF TABLES

Table

2-1. Characteristics of the mRNAs of the ovarian cytochrome P450 and their proteins 28

3-1. Frequency of follicles (dominant and subordinates) with P_4 concentrations <100 ng/ml or ≥ 100 ng/ml in the four experimental groups studied (day 5, E_2 active; D8 transitional E_2 active; D12-I atretic and D12+I E_2 active-sustained) 81

4-1. Frequency of follicles (dominant and subordinates) with E_2 concentrations <50 ng/ml or ≥ 50 ng/ml in the four experimental groups studied (day 5, E_2 active; D8 transitional E_2 active; D12-I atretic and D12+I E_2 active-sustained) 115

4-2. Simple (top right) and partial (bottom left) correlations between P_4, A_4 and E_2 FF concentrations (ng/ml) and steady state mRNA levels (arbitrary units/5μg total RNA/ follicle wall) of P450 SCC, P450 3βHSD, P450 17αHYD and P450 ARO enzymes in dominant follicles 127

4-3. Simple (top right) and partial (bottom left) correlations between P_4, A_4 and E_2 FF contents (ng/follicle) and mRNA contents (arbitrary units/total RNA/follicle wall) of P450 SCC, P450 3βHSD, P450 17αHYD and P450 ARO enzymes in DF 128

5-1. Frequency of follicles (dominant and subordinates) with E_2:P_4 ratio and E_2:A_4 ratio <1 or ≥ 1 in the four experimental groups studied (day 5, E_2 active; D8 non-E_2 active; D12-I atretic and D12+I E_2 active-sustained) 158

5-2. Simple (top right) and partial (bottom left) correlations between concentration of inhibin ($_{RIA}$INH; α-subunits, α-β dimers; ng/mg protein), amounts of 300, 196-116, 80, 49, 31 and 28 kDa inhibin forms ($_{IMB}$INH; α-subunits, α-β dimers; arbitrary units/10μg total protein) and steady state mRNA levels (arbitrary units/5μg total RNA) of α-inhibin and β_A-inhibin in dominant follicles 177

5-3. Simple (top right) and partial (bottom left) correlations between contents of inhibin ($_{RIA}$INH; α-subunits, α-β dimers; ng/follicle), contents of 300, 196-116, 80, 49, 31 and 28 kDa inhibin forms ($_{IMB}$INH; α-subunits, α-β dimers; arbitrary units/follicle) and mRNA contents (arbitrary units/follicle wall) of α-inhibin and β_A-inhibin in dominant follicles 178

6-1. Frequency of follicles (dominant and subordinates) with P_4:E_2 ratio <1 or ≥ 1 in the four experimental groups studied (day 5, E_2 active; D8 non-E_2 active; D12-I atretic and D12+I E_2 active-sustained) 214

6-2. Simple (top right) and partial (bottom left) correlations between concentrations of IGF-I, IGF-II (ng/ml), amounts of 35 kDa IGFBP-2, 49-43 kDa IGFBP-3, 28-22 kDa IGFBP-4 and 30 kDa IGFBP-5 ($_{LIGB}$IGFBP; arbitrary units/25μg total protein) and steady state mRNA levels (arbitrary units/5μg total RNA) of IGFBP-2 in dominant follicles 229

6-3. Simple (top right) and partial (bottom left) correlations between contents of IGF-I, IGF-II (ng/follicle), contents of 35 kDa IGFBP-2, 49-43 kDa IGFBP-3, 28-22 kDa IGFBP-4 and 30 kDa IGFBP-5 ($_{LIGB}$IGFBP; arbitrary units/follicle) and contents of IGFBP-2 mRNA (arbitrary units/follicle wall) in dominant follicles . 231

7-1. Summary of the six highest simple correlation coefficients found for each dependent variable studied 258

LIST OF FIGURES

Figure

2-1. Schematic representation generated from actual data for the growth and development of ovarian follicles during the estrous cycle of cattle (Savio et al., 1990c). 15

2-2. Schematic representation of ovarian steroid biosynthesis (Hinshelwood *et al.*, 1994) 21

2-3. Schematic representation of postranslational processing of inhibin/activin and follistatin molecules in bovine follicular fluid. . . . 40

3-1. Experimental details regarding design, estrus synchronization, ultrasonography, blood sampling and surgery of experimental animals 66

3-2. Pattern of growth of first wave dominant follicles (top-left), subordinate (top-right) and of dominance (difference in size between dominant and first subordinate follicle, bottom, center) during day 0 to day 12 of synchronized estrous cycles of non-lactating dairy cows 75

3-3. Number of class 1 (3-5 mm) follicles in period 1 (INT; both ovaries), period 1 (INT; average follicles per ovary) and period 2 (OVX) non-lactating dairy cows 76

3-4. Average diameter of the dominant and subordinate follicles (left) and average number of class 1 (3-5 mm), class 2 (6-9 mm) and class 3 (>10 mm; right) in D5, D8 and D12-I cows 77

3-5. Diameter of DF and SF at ovariectomy in non-lactating dairy cows. Data represent least squares means (±SEM) of follicle diameter in two periods (1=INT, 2=OVX; left), or in four experimental groups (D5, D8, D12-I and D12+I; right) 78

3-6. Plasma concentrations of P_4 during day 0 to day 12 of synchronized estrus cycles in dairy cows 80

3-7. Follicular fluid concentrations of P_4 in first wave DF and SF of dairy cows. 80

3-8. Northern blot analysis of P450 Side chain cleavage 83

3-9. Dot blot analysis of P450 Side chain cleavage 85

3-10. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 SCC cDNA to mRNA from first wave DF and SF of non-lactating dairy cows 86

3-11. Northern blot analysis of P450 3β-hydroxysteroid dehydrogenase 88

3-12. Dot blot analysis of P450 3β-hydroxysteroid dehydrogenase 90

3-13. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 3β-HSD cDNA to mRNA from first wave DF and SF of non-lactating dairy cows 91

4-1. Plasma concentrations of E_2 during day 0 to day 12 of synchronized estrous cycles in dairy cows 112

4-2. Follicular fluid concentrations of A_4 (left) and E_2 (right) in first wave DF and SF of dairy cows 114

4-3. Follicular fluid concentrations of E_2 in first wave DF of dairy cows 114

4-4. Northern blot analysis of P450-17αhydroxylase-17,20 lyase 117

4-5. Dot blot analysis of P450-17αhydroxylase-17,20 lyase 119

4-6. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 17α-HYD cDNA to mRNA from first wave DF and SF of non-lactating dairy cows 120

4-7. Northern blot analysis of P450 Aromatase 122

4-8. Dot blot analysis of P450 Aromatase 124

4-9. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 ARO cDNA to mRNA from first wave

DF and SF of non-lactating dairy cows 125

4-10. Standardized partial coefficients obtained from stepwise regression analyses of follicular fluid steroid concentrations and steroidogenic enzyme mRNA contents in whole dominant follicles 130

5-1. Follicular fluid E_2:P_4 ratio (EPR, left) and E_2:A_4 ratio (EAR, right) in first wave DF and SF of dairy cows. 156

5-2. Total inhibin radioimmunoactivity ($_{RIA}$INH; α-subunits, α-β dimer) concentrations in follicular fluid (ng/mg protein) in first wave DF and SF of dairy cows 159

5-3. Immunoblot analysis (α-subunits, α-β dimers) of non-reduced follicular fluid from representative first wave D5, D8, D12-I and D12+I dominant and subordinate follicles 161

5-4. Least square means (±SEM) of total $_{IMB}$INH intensity (α-subunits, α-β dimers; sum of arbitrary densitometric units for all forms [300, 196-116, 80, 49, 31 and 28 kDa] of inhibin/follicle) in first wave DF and SF of non-lactating dairy cows 162

5-5. Least square means (±SEM) of $_{IMB}$INH intensity (α-subunits, α-β dimers; arbitrary densitometric units of 300 [top-left], 196-116 [top-right], 80 [bottom-left], 49 [bottom-right] kDa forms of inhibin/follicle) in first wave DF and SF of non-lactating dairy cows 163

5-6. Least square means (±SEM) of $_{IMB}$INH intensity (α-subunits, α-β dimers; arbitrary densitometric units of 31 kDa (left) and 28 kDa (right) form of inhibin/follicle) in first wave DF and SF of non-lactating dairy cows 165

5-7. Least square means (±SEM) of proportion of $_{IMB}$INH intensity (α-subunits, α-β dimers; proportion of arbitrary densitometric units [AU] of each form [300, 196-116, 80, 49, 31 and 28 kDa] of inhibin/AU of total forms of inhibin) in first wave DF (left) and SF (right) of non-lactating dairy cows 167

5-8. Northern blot analysis of α-inhibin 168

5-9. Northern blot analysis of β_A-inhibin 171

5-10. Dot blot analysis of α-inhibin 172

5-11. Dot blot analysis of β_A-inhibin 173

5-12. Least square means ($\pm$SEM) of hybridization intensity (arbitrary units) of α-inhibin cDNA to mRNA from first wave DF and SF of non-lactating dairy cows 175

5-13. Least square means ($\pm$SEM) of hybridization intensity (arbitrary units) of β_A-inhibin cDNA to mRNA from first wave DF and SF of non-lactating dairy cows 175

5-14. Standardized partial coefficients obtained from stepwise regression analysis of follicular fluid $_{RIA}$INH contents, $_{IMB}$INH forms (300, 196-116, 80, 49, 31 and 28 kDa) contents, and inhibin α and β_A-subunit mRNA contents in whole dominant follicles 180

6-1. Follicular fluid P_4:E_2 ratio (PERT) in first wave DF and SF of dairy cows 213

6-2. Total protein concentrations (mg/ml) and total protein content (mg/follicle) in follicular fluid from first wave DF and SF of dairy cows 215

6-3. IGF-binding activity in Sep-Pak eluates of acidified bovine follicular fluid measured by charcol method 216

6-4. Recovery of added mass of IGF-I (left) and RIA displacement of [^{125}I]IGF-I (right) 217

6-5. Recovery of added mass of IGF-II (left) and RIA displacement of [^{125}I]IGF-II (right) 217

6-6. Follicular fluid IGF-I (left) and IGF-II (right) concentrations (ng/ml) in first wave DF and SF of dairy cows 219

6-7. Ligand blot analysis of follicular fluid from representative first wave D5, D8, D12-I and D12+I dominant and subordinate follicles . 220

6-8. Immunoprecipitation analysis of follicular fluid from a pool of representative first wave dominant (left) and subordinate (right) follicles 221

6-9. Least square means (±SEM) of total $_{LIGB}$IGFBP (sum of arbitrary units for all forms [49 and 43 kDa: IGFBP-3; 35 kDa:IGFBP-2; 30 kDa:IGFBP-5; 28 and 22 kDa:IGFBP-4] of IGFBP/follicle) in first wave DF and SF of non-lactating dairy cows 222

6-10. Least square means (±SEM) of $_{LIGB}$IGFBP intensity (arbitrary units of IGFBP-2 [35 kDa, top-left], IGFBP-3 [49 and 43 kDa, top-right], IGFBP-4 [28 and 22 kDa, bottom-left] and IGFBP-5 [30 kDa, bottom-right] per follicle) in first wave DF and SF of non-lactating dairy cows 224

6-11. Least square means (±SEM) of proportion of $_{LIGB}$IGFBP intensity (arbitrary units of IGFBP-2 [35 kDa], IGFBP-3 [49 and 43 kDa], IGFBP-4 [28 and 22 kDa] and IGFBP-5 [30 kDa] per follicle) in first wave DF and SF of non-lactating dairy cows 226

6-12. Dot blot analysis of IGFBP-2 227

6-13. Least square means (±SEM) of hybridization intensity (arbitrary units) of IGFBP-2 cDNA to mRNA from first wave DF and SF of non-lactating dairy cows 228

6-14. Standardized partial coefficients obtained from stepwise regression analysis of follicular fluid IGF-I and IGF-II content, $_{LIGB}$IGFBP (IGFBP-2, 35 kDa; IGFBP-3, 49 and 43 kDa; IGFBP-4, 28 and 22 kDa; IGFBP-5, 30 kDa) content and IGFBP-2 mRNA content in whole dominant follicles 233

7-1. Diagram of a theoretical model collating potential mechanisms involved in dominance of bovine ovarian follicles 261

7-2. Diagram of a theoretical model associated with potential mechanisms regulating atresia of bovine ovarian follicles 262

LIST OF ABBREVIATIONS

A_4	Androstenedione
AU	Arbitrary units
cAMP	Cyclic adenosine monophosphate
CAT	Chloranphenicol acetyl transferase
CIDR	Controlled internal drug release
cGMP	Cyclic guanidine monophosphate
CL	Corpora lutea
d	day
D5	Day 5
D8	Day 8
D12-I	Day 12 without progestin ear implant and prostraglandin $F_{2\alpha}$ injection at day 8
D12+I	Day 12 with progestin ear implant and prostraglandin $F_{2\alpha}$ injection at day 8
DE	Day of emergence
DF	Dominant follicle
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
E_2	17 β-estradiol
EA	Estrogen active
EI	Estrogen inactive
EAR	Estrogen-androstenedione ratio
EPR	Estrogen-progesterone ratio
FACS	Fluorescent activated cell sorting analysis
FF	Follicular fluid
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GC	Granulosa cells
hCG	human chorionic gonadotropin
HDL	High-density lipoprotein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGFBP	Insulin-like growth factor binding protein
IGFBPs	Insulin-like growth factor binding proteins
$_{IMB}INH$	Immunoblot inhibin

INT	Intact cow
IRMA	Two-site immunoradiometric assay
$_{\text{IRMA}}$INH	Immunoradiometric inhibin
Kb	Kilobases
kDa	Kilodalton
LDL	Low-density lipoproteins
LH	Luteinizing hormone
$_{\text{LIGB}}$IGFBP	Ligand blot insulin-like growth factor-binding proteins
LSM	Least square mean
M_r	Molecular weight
mRNA	Messenger ribonucleic acid
n	Number
OVX	Unulateral ovariectomized cow
P_4	Progesterone
P_5	Pregnenolone
P450 17α-HYD	Cytochrome P450-17αhydroxylase-17,20 lyase
P450 17β-HSD	Cytochrome P450 17β-hydroxysteroid dehydrogenase
P450 3β-HSD	Cytochrome P450 3β-hydroxysteroid dehydrogenase
P450 ARO	Cytochrome P450 aromatase
P450 SCC	Cytochrome P450 side chain cleavage
PERT	Progesterone-estradiol ratio
rRNA	Ribosomal ribonucleic acid
$_{\text{RIA}}$INH	Radioimmunoassay inhibin
SEM	Standard error of the mean
SF	Subordinate follicle
SPC	Standardized regression coefficients
T_4	Testosterone
TC	Theca interna cells
TGFβ	Transforming growth factor β

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ENDOCRINE AND MOLECULAR CHANGES ASSOCIATED WITH OVARIAN FOLLICULAR DOMINANCE IN CATTLE

By

Rodolfo Luzbel de la Sota

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To evaluate mechanisms controlling ovarian folliculogenesis in cattle, an experimental model was designed to obtain first wave estrogen active (E_2; day 5 [D5]), transitional E_2 active (D8), atretic (D12-I) and E_2 sustained (D12+I) dominant follicles (DF) and associated subordinate follicles (SF). Follicles were obtained by unilateral ovariectomy to measure concentrations of follicular fluid (FF) progesterone (P_4), androstenedione (A_4), E_2, insulin-like growth factors (IGF), inhibins by RIA, FF IGF-binding proteins (IGFBP) by ligand blot analysis, spectra of FF inhibins by immunoblot, and mRNA levels in follicle walls for the four ovarian steroidogenic enzymes, inhibins and IGFBP-2 by Northern and dot blot analysis. Ultrasonographic data and plasma P_4 and E_2 profiles were utilized to integrate endocrine and molecular findings with ovarian physiological status. Gene expression of steroidogenic enzymes decreased or increased with physiological status of the follicles but were unrelated to steroids in FF. Regulation of enzyme activity or mechanisms regulating enzyme translation or stability may play a more important role in regulating steroidal production during the transition from follicular dominance to atresia. Concentrations of FF inhibin measured by RIA and mRNA levels of α- and β_A-inhibin did not change markedly; however postranslational processing of high molecular weight (M_r) inhibin to low M_r inhibin molecules was associated with the transition from dominance to atresia or to sustained dominance. Two inhibin molecules were identified as potential candidates for exerting regulatory functions by either inhibiting FSH secretion (196-116 kDa) or by competing for the FSH receptor in SF (49 kDa). Although concentrations of IGF-I and II in FF did not change among DF and SF, induction of low M_r IGFBP (-2, -4 and -5) in FF of atretic DF and in most SF may reduce IGF-I stimulation of mitotic activity and decrease gonadotropin induced steroidogenesis in DF. Conversely, E_2 active sustained DF did not exhibit this array of low M_r IGFBP. A novel mathematical approach was utilized to determine potential interrelationships among measured responses in DF, and two theoretical models are presented to account for the different pathways of dominance and atresia of bovine follicles.

CHAPTER 1
INTRODUCTION

Bovine ovaries have a pool of primordial follicles, each consisting of an oocyte arrested in prophase I of meiosis and a single layer of undifferentiated squamous granulosa cells that develop during fetal life. Once this cohort of follicles has been established, follicles gradually and continually leave this resting pool to begin growth. This process of folliculogenesis is a continuous and a very selective process that culminates in atresia for the majority of the follicles, or selection of a dominant follicle that undergoes ovulation.

Follicular dynamics during the postpartum period and the estrous cycle are markedly altered by metabolic demands of lactation (de la Sota *et al.*, 1993; Lucy *et al.*, 1991a, 1993). In addition, hormonal and metabolic changes associated with lactation appear to alter terminal development of preovulatory follicles (de la Sota *et al.*, 1993). Thus, lactating cows have a lower degree of estrous intensity and are less fertile than non-lactating cows. Indeed, hormonal and metabolic responses associated with lactation attenuate follicular recruitment, and treatment with bovine somatotropin restores attenuated follicle development to a level similar to the non-lactating cow (de la Sota *et al.*, 1993). Furthermore, follicular dynamics also is altered by development of persistent follicles during the estrous cycle or in response to estrus synchronization protocols (Savio *et al.*, 1993a). In addition, follicular development may be potentially optimized to enhance follicular recruitment in superovulatory regimes utilized either in embryo transfer programs or in schemes where follicular aspiration, *in vitro* maturation and fertilization are utilized. Finally, distinct similarities exist between bovine and human species in ovarian follicular function. However, ovarian follicular dynamics and related endocrine events have not been thoroughly characterized in women due to practical and ethical limitations which prohibit gross, endocrine and histopathological verification of ultrasonographic observations made in humans *in vivo* (Adams and Pierson, 1995). Hence, newly developed techniques of controlling folliculogenesis in cattle may be modified appropriately for diagnostic and interventional procedures in women to augment or suppress fertility (Adams and Pierson, 1995).

Therefore, increasing basic knowledge of the mechanisms involved in controlling dominance and atresia of bovine follicles should contribute to an optimization of techniques used for estrus synchronization, treatment of ovarian follicular cysts, induction of superovulation, design of alternative nutritional management techniques that would hasten ovarian activity during the postpartum period and continued utilization of folliculogenesis in cattle as a model to develop new techniques to regulate fertility in humans.

The main objective of this research was twofold: to develop an experimental model to obtain first wave dominant and subordinate follicles at precise stages of follicle development (estrogen active, transitional estrogen active, atretic and sustained estrogen active); and to study three specific endocrine and molecular aspects that may regulate dominance and atresia of bovine ovarian follicles utilizing this model. These biological responses involved 1) steroid concentrations in follicular fluid and gene expression of steroidogenic enzymes within follicular walls; 2) concentrations of inhibin and amounts of different forms of inhibin detected in follicular fluid and inhibin gene expression within follicular walls; and 3) concentrations of IGF-I and -II concentrations and amounts of IGF-binding proteins in follicular fluid coupled with gene expression of IGFBP-2 within follicular walls.

CHAPTER 2
LITERATURE REVIEW

Ovarian Follicular Growth and Development

Ovarian Follicular Growth and Development in Embryonic Life

The primary sex organs develop from ridges on the ventromedial surface of the mesonephric kidneys (Latshaw, 1987; Noden and de Lahunta; 1985). The primordial germ cells and gonadal blastema compose the gonadal ridges of the undifferentiated embryonic gonads (Latshaw, 1987; Noden and de Lahunta; 1985). The primordial germ cells arise from endodermal cells of the yolk sack (Latshaw, 1987; Richards, 1980; Spicer and Echterncamp, 1986) and start migrating to the gonadal ridge through the mesentery around day 26 to 32 in cattle (Erickson, 1966; Latshaw, 1987). Although controversy exists regarding cell sources of the blastema, the current view is that blastema cells are primarily formed by mesonephric cells. Mitotic division of germ cells (i.e. oogonia) starts when germ cells arrive at the gonadal ridge, and the number of oogonia in embryonic gonads reaches a maximum around day 110 to 130 of fetal life (Erickson, 1966). In the bovine ovary, onset of meiosis occurs at approximately day 70 of embryonic life (Erickson, 1966; Latshaw, 1987; Marion and Gier, 1971); however, meiosis is not completed in the embryonic ovary and germ cells arrest in the diplotene stage of prophase

I. In cattle, most primary oocytes have achieved first meiotic arrest by day 170 to 175 of fetal life (Erickson, 1966) and remain in this stage until puberty.

Around day 90 to 100 of fetal life, the first primordial follicles are observed in the ovary of cattle (Erickson, 1966; Marion and Gier, 1971). At this time a single oocyte is surrounded by squamous, often irregularly shaped epithelial cells which more or less have separated completely the oocyte from cortical connective tissue (Erickson, 1966; Marion and Gier, 1972; Rajakoski, 1960). Eventually, the epithelial cells form a single layer of cuboidal cells which proliferate by mitosis and form a stratified follicular epithelium or granulosa layer with gap junctions. The follicle is termed a secondary follicle once cells surrounding the oocyte divide and form several layers. While these modifications take place, stroma immediately around the follicle differentiates to form the *theca folliculi*. This layer subsequently differentiates into theca interna and theca externa. As the follicle grows due to an increase in size and number of granulosa cells, accumulation of *liquor folliculi* appears between the granulosa cells. When the cavities that contain follicular fluid coalesce and form a single cavity, the *antrum*, these follicles are termed tertiary follicles (Latshaw, 1987). In the tertiary follicle (i.e. mature graafian follicle), cells of the granulosa layer form a small pedicle of cells, the *cumulus oophorus*, which contains the oocyte and protrudes towards the interior of the antrum. Granulosa cells that form a layer that surrounds the zona pellucida of the oocyte become elongated and form the *corona radiata*. The first antral follicles appear on the ovary approximately at 210 days of fetal life in cattle (Erickson, 1966).

Gonadotropin Receptors in Ovarian Follicles

Development of gonadotropin receptors on theca interna and granulosa cells is essential to the process of growth and development of ovarian follicles (Richards, 1980). Although there is enough evidence to indicate that no gonadotropin support is required to reach the stage of primordial and secondary follicle, antrum formation seems to be gonadotropin dependent (Richards *et al.*,1980). Hypophysectomy causes atresia of large antral follicles in the rat ovary after a short period of time (Richards, 1978). After several days of hypophysectomy, only primordial follicles are observed in the ovaries supporting the view that gonadotropin support is only required in large antral follicles. Granulosa cells of small antral follicles contain FSH receptors but no LH receptors (Findlay, 1993; Hillier, 1994; Magoffin and Erickson, 1994; Richards, 1980). *In vitro* studies support an autocrine role of activin promoting folliculogenesis during the preantral and early antral stages of follicle growth (Findlay, 1993). Activin treatment of cultured granulosa cells from DES-treated immature rats resulted in 4-fold increase in number of FSH receptor with no change in affinity (Xiao *et al.*, 1992). Thus ability of activin to upregulate FSH receptors in the absence of FSH suggests an unique role for activin in propagation of FSH receptors (Findlay, 1993). FSH stimulation induces LH receptor gene expression in theca interna cells (Segaloff *et al.* (1990). Ireland and Roche (1982) demonstrated the presence of LH receptors on theca cells of antral follicles. Granulosa cells from larger follicles contain receptors for both FSH and LH in cattle (Ireland and Roche, 1982), humans (Hillier, 1994) and rats (Magoffin and Erickson, 1994; Richards, 1980). Therefore, presence of both LH and FSH receptors in both cell types of antral follicles supports the two cell-two gonadotropin theory (Fortune and Amstrong, 1977,1978; Fortune and Hansel, 1979; Fortune, 1986). Thus, LH stimulates the synthesis of A_4 precursors in theca interna cells which are then aromatized to 17β-estradiol in granulosa cells under FSH stimulation.

Ireland and Roche (1982, 1983) characterized changes in growth, concentrations of steroids in FF and gonadotropin receptors of individual antral follicles after spontaneous luteolysis in heifers or in nonovulatory follicles after ovulation in heifers. Heifers were ovariectomized on days 3, 5, 7, 9, 11 and 13 of the estrous cycle and ovarian follicles greater than 6 mm were separated into two classes, EA and EI (Ireland and Roche, 1983). Estrogen active follicles had higher concentrations of E_2 than P_4 and low incidence of atresia whereas EI follicles had higher concentrations of P_4 than E_2 and a high incidence of atresia. The diameter of EA follicles, the volume and the number of granulosa cells per follicle increased from d 3 to d 7 and sharply decreased thereafter. On the other hand, on d3 of the estrous cycle, EI follicles had similar diameter, volume and number of granulosa cells per follicle compared to EA follicles. However, after d 3, EI follicles had one-third of the diameter and volume and one-forth the number of granulosa cells that EI follicles had. The specific binding of ^{125}IhCG to granulosa cells of EA follicles increased sharply from d 3 to d 7 and decreased thereafter; however, specific binding of ^{125}IhCG to theca cells reached maximum number at d 5, was maintained until d 7 and decreased thereafter. Specific binding of ^{125}IFSH was maximum at d5 in EA follicles and decreased thereafter. Maximum concentrations of FF E_2 were achieved at d5 of the estrous cycle and decreased very sharply thereafter to reach negligible concentrations at d 9. Furthermore, concentrations of E_2 in FF followed a similar pattern to that described for FSH and LH specific binding in theca and granulosa cells. Collectively, these data indicate that development of FSH and LH receptors in EA follicles is critical to develop of their steroidogenic capacity and give further support to the two cell two gonadotropin theory (Fortune and Amstrong, 1977,1978; Fortune and Hansel, 1979; Fortune, 1986). Hence, in concomitance with maximum E_2 production in the EA follicles at d5 of the estrous cycle, EA follicles have a maximum numbers of LH receptors in theca cells and FSH receptors in granulosa cells to support this function.

Studies of Bovine Follicular Development: Macroscopic and Microscopic Techniques

Before ultrasonic imaging became available in 1984 (Pierson and Ginther, 1984), growth and replacement rates, and the fate of bovine ovarian follicles during the estrous cycle were studied extensively utilizing a variety of macroscopic (Bherer *et al.*, 1976; Dufour *et al.*, 1972) and microscopic techniques (Choundary *et al.*, 1968; Dufour and Roy; 1985; Luissier *et al.*, 1987; Marion *et al.*, 1968; Matton *et al.*, 1981; Maurasse *et al.*, 1985; Rajakoski, 1960; Villeneuve *et al.*, 1988). Thirty-five years ago, Rajakoski (1960) proposed the existence of two waves of follicular growth. One wave culminated on day 12, followed by atresia whereas the other culminated at estrus. He described morphology of the ovarian follicular system and variations attributed to season, stage of the estrous cycle, asymmetry between right and left ovaries and corpus luteum development. Although each ovarian pair contained 1.6 normal follicles measuring ≥5 mm at any given day during the estrous cycle, the distribution of follicle numbers changed throughout the estrous cycle. One to three follicles (10-14 mm) were present per ovarian pair from day 1 and 2 up to ovulation. During days 3-4 of the estrous cycle, each pair of ovaries contained 3.4 follicles with an approximate diameter of 5 to 8 mm. Between days 5 and 11, only one follicle (9-13 mm) was present in each ovary pair. During days 12-14, each pair of ovaries contained 2.6 follicles (5-10 mm), and after day 15 to day 20 of the estrous cycle one follicle (8-13 mm) was present per ovarian pair. Rajakoski (1960) also concluded that the first wave dominant follicle that becomes atretic at day 12 of the estrous cycle remains on the ovary for another 5 days; that both ovaries had a similar number of follicles ≥ 1 mm; that the right ovary had a greater number of follicles ≥ 5mm, a greater frequency of ovulations, and a greater weight compared to the left ovary. Swanson *et al.* (1972) noticed similar changes in the number and size of follicles and agreed with Rajakoski's theory. Furthermore, Dufour *et al.* (1972) demonstrated that the largest follicle present on the ovary 5 days prior to estrus is not the follicle that ovulates. This provided the first evidence that turnover of the largest follicle in the ovary is quite rapid at the end of the cycle. Bherer *et al.* (1976) marked the two largest follicles present at day 10 or 14 of the estrous cycle with India ink and observed the fate of these follicles until ovulation. All largest and second largest follicles marked at day 10 of the estrous cycle became atretic whereas all largest follicles at day 14 of the estrous cycle ovulated. Matton *et al.* (1981) studied growth and replacement of bovine ovarian follicles during the estrous cycle. In an elegant experiment, they performed a series of laparotomies on four groups of 10 heifers at days 3, 8, 13 and 18 of the estrous cycle, and the number of large

(>6mm), medium-sized (3 to 6 mm) and small (1 to 3 mm) surface follicles were measured with a vernier caliper. In half of the animals, the two largest follicles were marked with India ink. In the remaining animals, all follicles greater than 5 mm were destroyed; and all animals were necropsied 5 days later. Results from this experiment provided additional evidence to indicate that growth of medium sized follicles only takes place when the largest follicle is either rapidly turning over or when destroyed. Contrary to the reports from Rajakoski (1960), Choundary *et al.* (1968) concluded that growth of follicles from one size to another was continuous and independent of stage of the estrous cycle. In this study the number of normal (non-atretic) follicles during early pregnancy was similar to the number during the luteal phase of the estrous cycle; however, a progressive decrease in the number of normal follicles was evident during latter stages of pregnancy.

Macroscopic studies in cattle by Dufour *et al.* (1972), Matton *et al.* (1981) and Pierson and Ginther (1984) provided evidence for growth and replacement rates of large follicles, but no information on small and medium size follicles. Marion and Gier (1971) suggested that growth of an antral follicle to the preovulatory size requires 40 days; recently Lussier *et al.* (1987) concluded that a period equivalent to two estrous cycles (42 days) is required for a follicle to grow through the antral phase (0.13 mm) to preovulatory size. The granulosa cell mitotic index was measured in non-atretic antral follicles at various diameters (0.13 to 8.57 mm) from Bouin-fixed ovaries before and after colchicine treatment (blocks metaphase stage of mitosis) to calculate duration of mitotic time, doubling time of granulosa cells, and the time in hours to pass through different size classes. Calculations based upon the number of granulosa cells in follicles of various classes and time required to double the number of cells within a follicle allowed Luissier *et al.* (1987) to conclude that it takes approximately 27 days for a follicle to grow from 0.13 to 0.67 mm, 6.8 days to grow from 0.68 to 3.67 mm and 7.8 days to grow from 3.68 to 8.56 mm; in follicles greater than 2.5 mm, follicular growth was the result of antrum development rather than an increase in the number of granulosa cells.

Studies of Bovine Follicular Development: Ultrasonic Imaging

Real-time ultrasonic imaging of ovarian structures provided a noninvasive and nondisruptive technique to image directly, *in situ*, daily changes in ovarian follicular dynamics during the estrous cycle in cattle (Pierson and Ginther, 1984; Ginther *et al.*, 1989ab; Savio *et al.*, 1988; Sirois and Fortune, 1988) and in other domestic species (reviewed by Griffin and Ginther, 1992). Briefly, ultrasound imaging utilizes high-frequency sound waves to produce images of the internal tissues and organs (Griffin and Ginther, 1992). Pulses of electric current produce the vibration of specialized piezoelectric crystals housed in the ultrasound transducer which generate sound waves. These sound waves sample a wide (e.g. 50 mm) but thin (e.g. 2 mm) tissue area which results in a two-dimensional image of the tissue. Thus, sound waves produced by the ultrasound transducer are directed through the tissue of interest by placing the transducer over the tissue and moving and/or varying the angle of the transducer. Ultrasonic characteristics of the tissue dictate the ability of the sound wave to reflect back to the transducer and be converted to electric current which are displayed as an echo on the ultrasound viewing screen. The echoes are displayed as varying shades of gray. Liquid does not reflect sound waves (nonechogenic or anechoic) and therefore are displayed as black images (e.g., ovarian follicle and embryonic vesicle) whereas dense tissues reflect a large proportion of the sound waves (echogenic) and hence are displayed on the viewing screen as light gray or white (e.g., pelvic bones, diestrous cervix). Transducers with three types of frequencies (i.e. 3.5, 5 and 7.5 MHZ) are used commonly for transrectal scanning of the reproductive tract of most large animals. Transducers with a higher frequency of transmitted sound waves have a shallower depth of penetration in the tissue and hence have a better resolution of the image. Therefore, a relatively small structure like a 5-15 mm follicle located close to the transducer generally are studied with a 5 or 7.5 MHz transducer. Conversely, transducers with lower frequency of transmitted sound waves have a lower resolution but a higher degree of penetration and therefore are more suited for studying large structures located far from the transducer such as a mid- or late gestation fetus and uterus.

Follicular Dynamics During the Bovine Estrous Cycle

Ultrasonic monitoring of ovarian follicles and corpora lutea have been used to study the follicular dynamics during the estrous cycle (Pierson and Ginther, 1984; Ginther *et al.*, 1989abc; Kastelic *et al.*, 1990a; Pierson and Ginther, 1987abc; Savio *et al.*, 1988; Sirois and Fortune, 1988), during pregnancy in heifers and cows (Ginther *et al.*, 1989d; Kastelic *et al.*, 1990a; Thatcher *et al.*, 1991) and during the early postpartum period (Lucy *et al.*, 1990; Savio *et al.*, 1990a; Rajamahendram and Taylor, 1990). Furthermore, ultrasonic imaging has been used to study the effects of prostaglandin $F_{2\alpha}$ on growth and regression of follicles either during the periovulatory period (Kastelic *et al.*, 1990b; Quirk *et al.*, 1986) or during the luteal phase (Savio *et al.*, 1990b), suppression of dominance by treatment with a proteinaceous fraction of follicular fluid (Kastelic *et al.*, 1990c) and temporal relationship between the FSH surge and initiation of a follicular wave (Adams *et al.*, 1992; Badinga *et al.*, 1992; Turzillo and Fortune, 1990). In addition, the effects of exogenous progestins (Savio *et al.*, 1993; Sirois and Fortune, 1990); estrogens (Bo *et al.*, 1993, 1994ab) ; GnRH agonists (MacMillan and Thatcher, 1991); hCG (Rajamahendram and Sianangama, 1992; Schmitt *et al.*, 1993); bovine somatotropin (de la Sota *et al.*, 1993); superstimulation (Adams *et al.*, 1994; Bungarts *et al.*, 1994; Guilbault *et al.*, 1991) and feeding different diets in fat and energy contents (Lucy *et al.*, 1993) on follicular dynamics have been studied by means of daily ultrasonic examination of the ovaries.

Although the first description of ultrasonic imaging to study the ovary in cattle was made by Pierson and Ginther (1984), the first studies of follicular dynamics in cattle were published almost four years later (Ginther *et al.*, 1989ab; Savio *et al.*, 1988; Sirois and Fortune, 1988). Before the utilization of ultrasonic imaging to study folliculogenesis in cattle, there was no agreement as to whether antral follicles were in a continuous state of turnover without any distinct pattern of growth and atresia during the estrous cycle (Chourdary *et al.*, 1968, Marion *et al.*, 1968; Dufour *et al.*, 1972) or if cows had two very distinct periods of turnover during the estrous cycle (Matton *et al.*, 1981; Rajakoski, 1960; Swanson *et al.*, 1972). Now it is well documented in cattle (Ginther *et al.*, 1989ab;

Savio *et al.*, 1988; Sirois and Fortune, 1988) that follicular growth is characterized by development of one or two dominant anovulatory follicles before the terminal preovulatory follicle is matured and ovulated. The first observation that follicular development in monoovulatory species proceeds through stages of recruitment, selection and dominance was made in primates by Goodman and Hodgen (1983) and later extended to cattle by Ireland and Roche (1987). Recruitment is the process whereby a cohort of antral follicles begins to grow under gonadotropin stimulation. Selection is a process by which one single follicle is chosen and avoids atresia. Dominance is the mechanism(s) by which a selected follicle with the potential competence to achieve ovulation inhibits recruitment of a new cohort of follicles. At early stages of the estrous cycle, a cohort of antral follicles is recruited out of a pool of smaller antral follicles (2 to 4 mm; Figure 2-1). The periovulatory FSH surges (Adams *et al.*, 1992; Turzillo and Fortune, 1990) and the presence of FSH receptors (Ireland and Roche, 1982) are thought to be responsible for recruitment of a cohort early in the estrous cycle. After 2 to 4 days of recruitment, one follicle is selected. Before selection takes place several medium-size follicles (6-9 mm) can be detected by ultrasonic imaging. However, after selection takes place a single follicle emerges from the pool of recruited follicles and continues to grow, whereas the remainder of the follicular cohort decrease in size. No single mechanism has been identified to explain the selection process; however, the prevalent concept is that once recruitment has started due to gonadotropin stimulation, the follicle that gets a head start in the coordinated gene expression of growth factors, steroidogenic enzymes and inhibins will be the selected follicle (Erickson *et al.*, 1994). Furthermore, once a follicle becomes

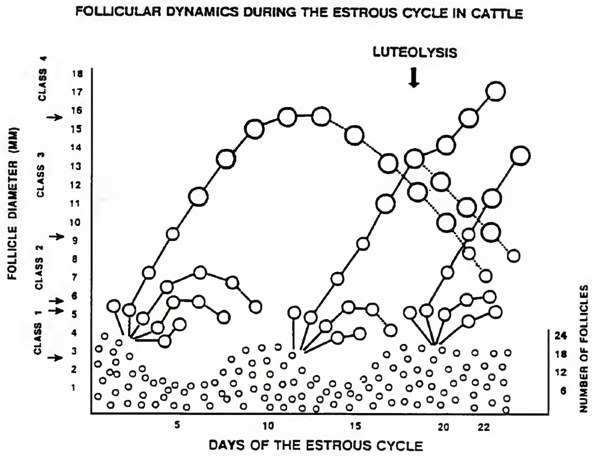

Figure 2-1. Schematic representation generated from actual data for the growth and development of ovarian follicles during the estrous cycle of cattle (Savio et al., 1990c). Follicular size classes are indicated on ordinate (Class 1 = 3 to 5 mm, Class 2 = 6 to 9, Class 3 = 10 to 15 mm, Class 4 = >15 mm).

selected it suppresses growth of cohort follicles and inhibits further recruitment of new follicles (Adams *et al.*, 1991; Savio *et al.*, 1993a). The first wave dominant follicle increases in size to approximately day 8, enters a plateau phase between days 8 and 10 of the estrous cycle, and then gradually starts to decline in size (Figure 2-1). At this time (day 10-12 of estrous cycle), dominance of the first dominant follicle is arrested, and recruitment of the second wave follicle begins. Mechanisms that regulate first wave dominant follicle turnover are poorly understood. However, it has been hypothesized that the increase in plasma progesterone concentrations produced by the newly formed corpus luteum is responsible for a decrease in LH pulse frequency (Rahe *et al.*, 1980; Savio *et al.*, 1993a) and that inadequate LH support to the dominant follicle induces atresia. After regression of the first wave dominant follicle, a second wave of follicle recruitment, selection and dominance takes place (Ginther *et al.*, 1989ab; Savio *et al.*, 1988; Sirois and Fortune, 1988). Final maturation of the second dominant follicle of the estrous cycle is associated with regression of the CL and ovulation. Alternatively, the second dominant follicle may become atretic, and, if this occurs, a third wave of follicle development is initiated. In cows with three waves of follicle development, size of the second wave dominant follicle is smaller than the size of the first wave dominant follicle (Sirois and Fortune, 1988; Savio *et al.*, 1993a; Taylor and Rajamahendran, 1991), and length of the estrous cycle is extended because the third dominant follicle requires additional time to complete development before ovulation (Savio *et al.*, 1993a; Taylor and Rajamahendran, 1991). Although most cows have either two or three waves of follicular growth, estrous cycles with four waves of follicle growth have been reported (Savio *et al.*, 1988; Sirois and Fortune, 1988).

One of the major advantages of ultrasonic imaging for studying follicular dynamics is the daily characterization of follicle number and size in each ovary. Furthermore, daily recording of images from the scanning with a video recorder and/or drawing of ovarian maps containing size and relative position of follicles greater than 3 mm and corpora lutea (Pierson and Ginther, 1984) facilitates later analysis and interpretation of collected data (de la Sota *et al.*, 1993; Lucy *et al.*, 1992). During early stages of the estrous cycle, number of class 1 (3-4 mm) follicles decrease while the number of class 2 follicles (5-9 mm) increase. The increase in the number of class 2 follicles represents recruitment from a group of class 1 follicles during the recruitment phase. On approximately day 4 or 5 of the estrous cycle, one follicle becomes selected and enters into class 3 (follicles ≥ 10 mm). At the same time, the number of class 2 follicles starts to decline because the cohort of follicles that is not selected starts to undergo atresia and therefore decrease in size. The number of follicles remains unchanged until day 10 to 12 of the estrous cycle when the first wave dominant follicle regresses and loses dominance and a new wave of follicle recruitment is initiated (Lucy *et al.*, 1992).

Experimental Models Utilized to Study Follicular Dynamics During the Bovine Estrous Cycle

Follicles obtained from ovaries collected from abattoirs have been used in the past as a source to obtain ovarian follicles for studying endocrine and molecular aspects of dominance and atresia in cattle (Grimes and Ireland, 1986; Grimes *et al.*, 1987; Ireland *et al.*, 1994; Ireland and Ireland, 1994). Stage of the estrous cycle is dated by appearance of the CL (Ireland *et al.*, 1980) . However, the main limitation from using this source of follicles is that physiological stages of follicles and animal are unknown and/or inprecise. An experimental model for obtaining follicles at precise stages of the estrous cycle provides a better opportunity to integrate plasma steroids and ultrasonic imaging data with follicular fluid responses and tissue expression of genes deemed important for follicular function. De la Sota *et al.* (1993) studied the effects of bovine somatotropin on growth and development of follicles during the first follicular wave and the preovulatory wave of estrous cycles "programmed" to have only two follicular waves. In order to program estrous cycles to have two follicular waves, cows were injected with $PGF_{2\alpha}$ to induce corpus luteum regression on day 12 of the estrous cycle and a CIDR device containing 1.9 g of progesterone was inserted into the vaginas of all cows. Five days later (day 17), CIDR devices were removed and the follicular wave ended when cows expressed estrus and ovulated. Sirois and Fortune (1990) utilized a model where the luteal phase was extended with low levels of exogenous progesterone to promote development of the dominant follicle and hence study potential regulators of follicular dominance in cattle. More recently, Voss and Fortune (1993ab) utilized the preovulatory follicle as an experimental model to study steroidogenic gene expression before and after the LH surge. Badinga *et al.* (1992) utilized the first wave dominant follicle as an experimental model to study endocrine and biochemical differences among dominant and subordinate follicles on days 5, 8 and 12 of the estrous cycle. To study sustained dominance in cattle, Savio *et al.* (1993a) developed a model where turnover and atresia of the first wave dominant follicle was prevented for a 15 day period by induction of luteolysis and insertion of a progestin implant on day 8 of the estrous cycle. Collectively, follicle development in cattle during the estrous cycle proceeds through the sequential stages of recruitment, selection and dominance. Although two or three waves of follicle development occur during the bovine estrous cycle, development of the first dominant follicle appears to be the most consistent and predictable event related to follicular dynamics during the bovine estrous cycle. Therefore, the first wave dominant follicle has the potential to be utilized as an experimental model for studying endocrine and molecular aspects of follicular dominance and atresia in cattle.

Steroidogenic Enzymes and Steroid Hormones

Steroidogenesis in Bovine Nonovulatory and Preovulatory Follicles

Steroidogenesis in the bovine preovulatory follicle requires uptake or *de novo* synthesis of cholesterol and conversion of cholesterol to P_5 by P450 SCC (Miller, 1988). This step takes place in the mitochondria and is the first and rate-limiting step in the synthesis of steroid hormones in ovarian follicles (Hanukoglu, 1992; Miller, 1988). The conversion step is done by three successive monooxygenations of cholesterol (hydroxylations at C-22, followed by C-20, and finally cleavage of the C-20,22 bond). Whereas P450 SCC is expressed in theca interna cells, its expression in granulosa cells depends on stage of follicle development.

Pregnenolone is converted subsequently into 17-hydroxy-pregnenolone and DHEA through the Δ^5 pathway or into P_4 by P450 3β-HSD and subsequently converted to 17-

hydroxy-progesterone and A_4 through the Δ^4 pathway (Hanukoglu, 1992; Miller, 1988). In both pathways, the conversion of either P_5 to DHEA or P_4 to A_4 is accomplished by the enzyme P450 17α-HYD; and P450 3β-HSD converts 17-hydroxy-pregnenolone to 17-hydroxy-progesterone and DHEA to A_4 (Hanukoglu, 1992; Miller, 1988). Whereas the Δ^5 pathway is the predominant pathway for the production of A_4 in cattle and humans, the Δ^4 pathway is the predominant pathway for the production of A_4 in rats, mares and sows (reviewed by Lacroix *et al.*, 1974). In granulosa cells, A_4 acts as the predominant substrate for aromatization by P450 ARO and is converted to E_2 supporting the concept of the two cell theory (Falk, 1959; Fortune, 1986; Hanukoglu, 1992; Lacroix *et al.*, 1974). A schematic representation of the ovarian steroid biosynthesis is depicted in Figure 2-2 (Hinshelwood *et al.*, 1994). In summary, each of the hydroxylation reactions catalyzed by the cytochrome P450 enzymes is characterized by the subsequent removal of either six carbons (P450 SCC), two carbons (P450 17α-HYD) or one carbon (P450 ARO). In addition, P450 3β-HSD catalyzes the oxidation of the hydroxyl at C3 and the subsequent isomerization of Δ^5 steroids to Δ^4 products. The reversible conversion of A_4 to testosterone (T_4) is a consequence of the enzyme P450 17β-HSD present only in theca interna cells (Hanukoglu, 1992).

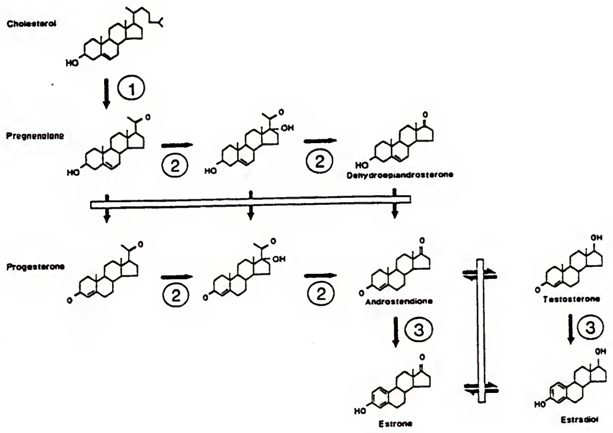

Figure 2-2. Schematic representation of ovarian steroid biosynthesis (Hinshelwood *et al.*, 1994). ①, P450 SCC; ②, P450 17 α-HYD; ③, P450 ARO. Horizontal bar is P450 3 β-HSD, vertical bar is P450 17 β-HSD.

Two-Cell, Two-Gonadotropin Theory

One of the first experiments designed to study the source of steroids in ovarian follicles was done 35 yr ago by Falk (1959). Utilizing autotransplanted ovarian tissue to the anterior chambers of the eye of spayed rats and using contiguous vaginal autotransplants as an indicator of estrogenic activity of the ovarian grafts, Falk's results were the first to show critical experimental evidence that follicular estradiol production was dependent on an interplay between theca interna and granulosa cells. In these series of experiments, three combinations of autotransplants were used: interstitial cells and corpus luteum cell, theca interna cells and granulosa cells, and theca interna cells and corpus luteum cells. The only combination that induced estrogen dependent changes in the contiguous vaginal autotransplant was the theca interna cells and granulosa cells in combination supporting the hypothesis that estrogen was locally produced in the follicle and was dependent on an interaction between theca interna and granulosa cells.

Further support for the two cell model for estradiol production came almost 15 yr later with experiments done by Fortune and Amstrong (1977,1978). In these experiments, theca and granulosa cells from proestrous rats were cultured in the presence or absence of exogenous gonadotropins and with or without exogenous aromatizable precursors. Theca interna cells secrete A_4 under LH stimulation but possess very little or no capability to aromatize A_4 to E_2 (Fortune and Amstrong, 1978). Conversely, GC are capable of aromatizing exogenous A_4 to E_2 (Fortune and Amstrong, 1978) but are unable to synthesize A_4 (Fortune and Amstrong, 1977).

Quite similar results were obtained in experiments with theca and granulosa cells isolated from bovine proestrous follicles (Fortune, 1986). These experiments showed that bovine TC secrete A_4 under LH but not under FSH stimulation (Fortune, 1986), and although GC secrete P_4, these granulosa cells are incapable of converting it to E_2 (Fortune and Hansel, 1979). Granulosa cells could only secrete E_2 when an aromatizable androgen was included in the culture medium (Fortune and Hansel, 1979). Hence these series of studies provide enough evidence to extend the two-cell model for E_2 to ovarian follicles in cattle. Theca cells are stimulated to produce A_4 under LH stimulation but are unable to convert A_4 to E_2. Conversely, as GC are not able to synthesize A_4 *de novo*, GC aromatize TC produced A_4 to E_2. Additional studies from the same laboratory provided evidence to indicate that GC indirectly contribute to TC A_4 production by providing P_5 which is converted to A_4 in TC (Fortune, 1986). These results contrast to those obtained from rat cell cultures where P_4 is the more effective substrate for A_4 (Fortune, 1986). These results indicate that even though TC of both rats and cattle use progestin precursors for A_4 production, rats preferentially use the Δ^4 pathway (P_4 to A_4); and cattle use the Δ^5 pathway (P_5 to A_4).

Steroidogenesis in bovine theca and granulosa cells has one additional and distinct characteristic. Estradiol causes positive feedback on its own production by inhibiting P_4 secretion in theca and granulosa cells (Fortune and Hansel, 1979) and increasing P_5 production in GC. This inhibitory effect appears to be mediated through inhibition of P450 3β-HSD that converts P_5 to P_4. It is noteworthy to mention that P450 3β-HSD also converts DHEA to A_4 in TC; however, this step does not appear to be inhibited by GC E_2.

Further support for this selective inhibition comes from experiments where TC were exposed to epostane which is a P450 3β-HSD inhibitor. Results from these experiments showed that epostane selectively inhibited P_4 but did not affect A_4 secretion (Fortune and Quirk, 1988).

Steroid Concentrations in Follicular Fluid: Indices of Dominance and Atresia

Absolute concentrations of FF steroids and/or absolute ratios of FF steroids (Badinga, 1992; Grimes and Ireland, 1986; Grimes *et al.*, 1987; Guilbault *et al.*,1993; Ireland and Roche, 1982,1983; Ireland *et al.*,1994; Ireland and Ireland, 1994; Martin *et al.*, 1991; McNatty *et al.*,1984; Voss and Fortune, 1993ab), macroscopic appearance of the surface of the follicle (Grimes and Ireland, 1986), comparison of histological and non-histological indices of atresia (Grimes *et al.*, 1987; Guilbault *et al.*,1993) and ultrasonographic data (Badinga *et al.*, 1992; Guilbault *et al.*,1993) have been utilized to classify individual follicles as non-atretic (E_2 active) and atretic (E_2 inactive) in cattle. Follicular fluid collected from ovarian follicles during the first wave of follicle development (Ireland and Roche, 1983; Martin *et al.*, 1991; Badinga *et al.*, 1992) were classified as either non-atretic (EA; EPR > 1; PERT < 1) and atretic (EI; EPR < 1; PERT > 1) based upon the absolute ratio of FF steroids. In addition, absolute concentrations of FF steroids also have been utilized to classify individual follicles as non-atretic or atretic. Using absolute FF E_2, Ireland and Roche (1983) classified bovine follicles as non-atretic (EA; $E_2=141\pm35$ ng/ml) or atretic (EI; $E_2=8\pm2$ ng/ml) whereas Ireland and Ireland (1994) classified bovine follicles as non-atretic (EA; $E_2=99\pm32$ ng/ml), atretic (EI;

$E_2 = 19 \pm 15$ ng/ml) and highly atretic (EI; $E_2 = 0.1 \pm 0.02$ ng/ml). Echternkamp *et al.* (1994) used a threshold of 50 ng/ml of E_2 in FF to classify porcine follicles as either non-atretic or atretic. In addition, absolute P_4 concentrations in FF have been used to classify individual follicles as highly atretic, atretic or non-atretic. Hence, Ireland and Roche (1983) classified bovine follicles as atretic (EI; $P_4 = 108 \pm 25$ ng/ml) or non-atretic (EA; $P_4 = 36 \pm 5$ ng/ml) whereas Ireland and Ireland (1994) classified bovine follicles as highly atretic (EI; $P_4 = 516 \pm 113$ ng/ml), atretic (EI; $P_4 = 66 \pm 48$ ng/ml) and non-atretic (EA; $P_4 = 20 \pm 3$ ng/ml). In the past, histological studies have been used alone or in conjunction with FF steroid profiles to classify bovine follicles as atretic or non-atretic (Grimes and Ireland, 1986; Grimes *et al.*, 1987; Guilbault *et al.*,1993; Ireland and Roche, 1983). The presence of mitotic figures, integrity of at least 95% of granulosa cells against the basement membrane, and the presence of $< 1\%$ of pycnotic GC have been set as the three responses to be fulfilled by a follicle to be classified as non-atretic.

Daily ultrasonographic observation of bovine ovarian follicles has permitted the identification of the dominant follicle and the cohort of subordinate follicles in bovine ovaries as early as day 4 of the estrous cycle (Badinga *et al.*, 1992; de la Sota, 1993; Ginther *et al.*, 1989abc; Guilbault *et al.*, 1993; Lucy *et al.*, 1992; Savio *et al.*, 1988,1990; Sirois and Fortune, 1988). The continued suppression of a new cohort of subordinate follicles collated with the profile of growth or regression of the second largest follicles has been used to assess the functional status of the morphologically dominant follicle (Guilbault *et al.*, 1993). Therefore, a combination of ultrasonography, absolute FF concentrations of steroids or the ratio of FF steroids has provided the opportunity to classify more accurately follicles obtained after unilateral ovariectomy in cattle (Badinga *et al.*, 1992). Collectively, these studies indicate that bovine ovarian follicles can be classified as non-atretic or atretic based upon the following characteristics: 1) EPR >1 or ≤ 1, 2) PERT ≤ 1 or >1, 3) threshold of FF P_4 (100 ng/ml) or E_2 (50 ng/ml) and ultrasonic imaging data (size and day of wave of DF and SF), and 4) histological indices and combination of the three previous criteria.

Profiles of FF steroids have been measured in individual ovarian follicles during the first wave of follicle development (Badinga, *et al.*, 1992; Guilbault *et al.*,1993; Ireland and Roche, 1983, Martin *et al.*, 1991), during the preovulatory period (Fortune and Hansel, 1985; Ireland and Roche, 1982, Martin *et al.*, 1991; McNatty *et al.*, 1984; Voss and Fortune, 1993ab), or in ovarian follicles obtained from the abattoir (Grimes and Ireland, 1986; Grimes *et al.*, 1987; Ireland *et al.*, 1994; Ireland and Ireland, 1994). During the first wave of follicle development, FF A_4 and E_2 concentrations increase to reach their highest levels around d5 and then start to decrease to very low levels around d 12 in atretic follicles. Conversely, FF P_4 concentrations increase with atresia.

Steroidogenic Enzyme Gene Expression in Ovarian Follicles

One of the most important findings to emerge from molecular studies of steroidogenic enzyme gene expression in the human, cow, pig and rat is that all ovarian steroidogenic enzymes except P450 3β-HSD are encoded by only one gene (Hanukoglu, 1992). Although in humans, rats and mice, there are at least 2-3 homologous genes encoding for P450 3β-HSD that share 80-94% sequence identity within species, in cows there is only one gene (Labrie *et al.*,1992). The characteristics of the mRNAs of the ovarian cytochrome P450 and their proteins are depicted in Table 2-1. With the only exception of P450 ARO aromatase mRNA, the other mRNAs and proteins do not change significantly across species (Table 2-1).

Levels of mRNA, protein and activity of P450 SCC (Funkenstain *et al.*, 1983, 1984; John *et al.*, 1984; Hinshelwood *et al.*, 1993; Rodgers *et al.*, 1986ab, 1987) and P450 3β-HSD (Couet *et al.*, 1990; Zhao *et al.*, 1989) have been measured in bovine follicles and corpora lutea from ovaries obtained at the abattoir. Funkenstain *et al.* (1983, 1984) investigated the actions of FSH and cAMP analogs to stimulate production of P_4 and the synthesis of P450 SCC in bovine granulosa cells in monolayer culture. Treatment of granulosa cells in culture with either FSH or cAMP analogs resulted in an increase in immunoprecipitable P450 SCC, a higher rate of enzyme synthesis, and stimulation of P_4 production when compared to control cell cultures. More recently, Rodgers *et al.* (1986a) studied the immunolocalization of P450 SCC in bovine follicles (n=25; >3 mm in diameter) from ovaries obtained at the abattoir. Positive immunostaining for P450 SCC was localized in cells of the theca interna of large follicles and of follicles undergoing atresia. In addition, positive staining was present in granulosa cells of large follicles. In another study, Rodgers *et al.* (1986b) utilized immunoblot analysis to determine amounts of P450 SCC enzyme in tissue homogenates from medium sized (9-11 mm) and large (14-18 mm) follicles as well as CL from early, early-mid, late-mid, and late stages of the

Table 2-1. Characteristics of the mRNAs of the ovarian cytochrome P450 and their proteins[1].

P450 Enzyme	Species	mRN length (kb)	Protein length aa[a] (preseq)[b]	References
SCC[c]	Human	2	482 (521)	Solish *et al.*, 1988; Chung *et al.*,1986; Moore *et al.*, 1990; Morashashi *et al.*, 1987
	Rat	2	490 (526)	McMasters *et al.*, 1987; Oonk *et al.*, 1989
	Cow	2	481 (520)	John *et al.*, 1984; Morashashi *et al.*, 1984; Ahlgren *et al.*, 1990
	Pig	-	481 (520)	Mulheron *et al.*, 1989
3 β-HSD[d]	Human (2)	1.7	371, 372	Labrie *et al.*, 1992
	Rat (3)	1.7	372	Labrie *et al.*, 1992
	Cow	1.7	372	Zhao*et al.*, 1989
17 α-HYD[e]	Human	1.9	508	Sparkes *et al.*, 1991; Bradshaw *et al.*,1987; Chung *et al.*, 1987; Picardo *et al.*, 1987
	Rat	1.9	507	Fevold *et al.*, 1989; Namiki *et al.*, 1988
	Cow	1.9	509	Brasker *et al.*, 1989; Zuber *et al.*, 1986
ARO[f]	Human	2.9, 3.4	503	Chen *et al.*, 1988; Harada *et al.*, 1988; Simpson *et al.*, 1987; Toda *et al.*, 1990
	Rat	2.7, 2.2, 1.7	508	Hickey *et al.*, 1988, 1990
	Cow	6.0	503	Hinshelwood *et al.*, 1993
	Pig	3.4	-	Guthrie *et al.*, 1994

[1] Compiled from: Hanukoglu, 1992; Hinshelwood *et al.*, 1993; Labrie *et al.*, 1992
[a] amino acids, [b]presequence of the protein includes amino terminal extension signal peptide,
[c] P450 side-chain cleavage, [d] P450 3 β-hydroxysteroid dehydrogenase/ Δ^5- Δ^4-isomerase,
[e] P450 17- α-hydroxylase/C-17,C20 lyase, [f] P450 aromatase.

luteal phase (n=5/group). The specific contents of P450 SCC were similar between follicles and CL of early luteal phase; however, by early-mid luteal phase the specific contents of P450 SCC were increased 12-fold. Relative levels of mRNA specific for P450 SCC have been studied in bovine follicles and CL obtained from ovaries obtained at abattoir (Rodgers *et al.*, 1987; Hinshelwood *et al.*, 1993). In both studies, mRNA levels of P450 SCC were higher in CL than in all follicles studied. Changes in P450 3β-HSD mRNA, activity and protein levels during the bovine estrous cycle also were studied by Couet *et al.* (1990) in follicles and CL obtained from the abattoir. Sixteen follicles smaller than 4 mm and eight follicles greater than 4 mm in diameter were utilized. A parallel increase in P450 3β-HSD mRNA, protein content and enzymatic activity levels from days 1-3 after estrus to maximal values at 50 to 100% above control (i.e. d 0) on days 8-11 after estrus were observed. Thereafter, all values decreased progressively until days 16-17 before a dramatic fall to 5% or less of maximal values on days 18-20 after estrus. Ovarian follicular activity of P450 3β-HSD was approximately 10,000 times lower than that measured in CL. More recently, follicles obtained from ovaries at precise stages of the estrous cycle were utilized for studies of P450 SCC and P450 3β-HSD mRNA levels during the periovulatory period (Voss and Fortune, 1993a) or at different stages of follicle development during the first 10 days of the first follicular wave (Xu *et al.*, 1994). Bovine preovulatory follicles were obtained in the early follicular phase, mid follicular phase, or late follicular phase (after the LH surge, but before ovulation; Voss and Fortune, 1993a). Levels of P450 SCC mRNA did not change with stage of follicle development in theca interna cells; however, levels decreased by 96% in granulosa cells after the LH surge.

P450 3β-HSD mRNA levels were 3.6- and 2.6-fold higher in the early vs the mid and late follicular phase theca interna cells; and were 4.2-fold higher in early vs late follicular phase in granulosa cells. Progesterone concentrations in FF increased linearly from early to mid to late follicular phases after the LH surge. The authors concluded that the increase in production of P_4 by theca interna and granulosa cells could not be explained by an increase in message of P450 SCC and P450 3β-HSD, and therefore suggested that the increase in FF P_4 between the LH surge and ovulation resulted from either a decreased capacity to metabolize progestins to androgens and/or the continued presence of both enzymes in an active form. Xu *et al.* (1994) used *in situ* hybridization techniques to study gene expression of mRNA for P450 SCC in bovine follicles obtained at 0, 2, 4, 6, 8 and 10 days after initiation of the first wave of follicle development. P450 SCC mRNA was localized in the theca interna of follicles at d 2 and d 4 of the estrous cycle and in granulosa cells of follicles >4 mm. However, in this study steroid concentrations in follicular fluid and the stage of follicle development (dominant vs atretic) were not available to allow establisment of relationships with gene expression.

Levels of mRNA, protein and activity of P450 17α-HYD (Hinshelwood *et al.*, 1993; Rodgers *et al.*, 1986ab, 1987) and P450 ARO (Hinshelwood *et al.*, 1993) have been measured in bovine follicles and corpora lutea from ovaries obtained at the abattoir. Positive immunostaining for P450 17α-HYD has been localized in theca interna cells and in granulosa cells of large follicles (>15 mm; Rodgers *et al.*, 1986a). In another study, levels of P450 17α-HYD enzyme were measured by immunoblot analysis in medium (9-11 mm) and large (14-18 mm) bovine ovarian follicles and in corpora lutea from early, early-mid, late-mid and late stages of luteal phase (n=5/group; Rodgers *et al.*, 1986b). The specific tissue contents of P450 17α-HYD enzyme increased 5-fold from medium to large follicles, but were in corpora lutea at all stages of the luteal phase. P450 17α-HYD mRNA levels followed a similar pattern of expression as did enzyme contents (Rodgers *et al.*, 1987; Hinshelwood *et al.*, 1993). Gene expression of P450 17α-HYD was higher in large follicles compared to medium size follicles, and levels were very much decreased in young CL and undetectable in mature CL. More recently, bovine preovulatory follicles obtained in either the early follicular phase, mid follicular phase, or late follicular phase (after the LH surge, but before ovulation) were utilized for studies of P450 17α-HYD and P450 ARO mRNA expression (Voss and Fortune, 1993b). Messenger RNA levels for P450 17α-HYD and P450 ARO were highly abundant before the LH surge, but decreased by 96 and 94% respectively after the LH surge. Similar patterns were observed with FF concentrations of A_4 and E_2. The authors concluded that the dramatic decrease in FF steroids could be in part accounted by the decrease in mRNAs for both steroidogenic enzymes. A different approach was undertaken by Xu *et al.* (1994). *In situ* hybridization techniques were utilized to study gene expression of mRNA for P450 17α-HYD and P450 ARO in bovine follicles obtained at 0, 2, 4, 6, 8 and 10 days after initiation of the first wave of follicle development. In this study, hybridization for P450 17α-HYD was localized only in theca interna cells whereas hybridization for P450 ARO was localized only in granulosa cells. Levels of both enzymes were maximum at day 4 and 6 of the first wave of follicle development and thereafter decreased to undetectable levels. In summary, several studies addressed the qualititative and quantitative changes in gene expression of these four ovarian steroidogenic enzymes in follicles obtained at different stages of follicle development. However, these follicles generally were obtained from ovaries collected at the abattoir and hence stage of follicle development was not precise nor was a description of the steroid concentrations given. Conversely, the study reported by Voss and Fortune (1993ab) was done with follicles obtained at precise stages of development and steroid profiles were available; however, because their experimental model utilized the periovulatory dominant follicle rather than the first wave dominant follicle, changes in expression of ovarian steroidogenic enzymes during turnover and atresia of follicles were not studied. Hence the study of gene expression and FF steroid concentrations utilizing follicles obtained during the first wave of follicle development has the potential to provide this information.

Regulation of Steroid Hormones Production at the Ovarian Level

Steroid hormone output at the ovarian level depends on the rate of steroid biosynthesis and catabolism (Hanukoglu, 1992). The pathway for steroid hormone biosynthesis starts with cholesterol derived primarily from circulating lipoproteins (i.e. HDL, LDL) rather than from *de novo* synthesis from acetate (Hinshelwood *et al.*, 1994). In cattle and sheep, addition of either HDL or LDL increases P_4 production in cultured luteal cells (Carrol *et al.*, 1992; Wiltbank *et al.*, 1990). Gonadotropin hormones activate a series of reactions that leads to the hydrolysis of cholesterol and transport of cholesterol into the mitochondria. Conversion of cholesterol to pregnenolone in mitochondria by P450 SCC is the first, rate-limiting and hormonally regulated step in the synthesis of all steroid hormones (Hanukoglu, 1992; Miller, 1988). Subsequent biosynthetic steps proceed with the flow of substrates through enzymes located in the endoplasmic reticulum (Miller, 1988). Furthermore, steroid hormones are hydrophobic molecules that can penetrate biological membranes, and can flow pass into the blood stream after being synthesized without being stored in intracellular vesicles.

Steroid hormone production is regulated by at least four different mechanisms (Hanukoglu, 1992): 1) mRNA levels of steroidogenic enzymes as determined by transcription, stability and translation of mRNAs encoding the enzymes; 2) steroidogenic enzyme activity as determined by postranslational modifications of the enzymes by cofactor availability and by conditions of the intracellular milieu; 3) substrate availability, and 4) tissue growth as, determined by cell division and multiplication (i.e., growth of dominant follicle and corpus luteum). Gonadotropin stimulation of granulosa and theca cells in culture generates an increase of mRNA for steroidogenic enzymes within a few hours, and reach maximum values at 6-12 hrs post stimulation (Steinkampf *et al.*, 1987; Funkenstain *et al.*, 1984; Veldhuis and Rodgers, 1987). Furthermore, when mRNA levels start to drop, levels of enzyme do not a show similar decrease, probably reflecting the fact that the enzymes have much longer half lives than mRNAs (Hanukoglu, 1992). The "two cell, two gonadotropin" theory for E_2 synthesis in ovarian follicles, states that LH stimulates the synthesis of A_4 precursors in theca interna cells whereas FSH stimulates aromatization of A_4 in granulosa cells (Fortune, 1986; Hillier, 1994; Magoffin and Erickson, 1994). Compelling evidence demonstrating the effects of FSH and LH on ovarian steroidogenesis has become available in the last decade. The interaction of FSH or LH with the granulosa and theca internal cell receptors activate adenylate cyclase and phospholipase C, causing a transient increase in intracellular levels of second messengers such as cAMP, cGMP, inositol triphosphate, Ca^{2+} and diacylglycerol (Leung and Steele, 1992). Thereafter, cAMP activates protein kinase A, and Ca^{2+} and diacylglycerol activate protein kinase C; both kinases phosphorylate proteins and hence affect their function. The list of FSH responsive genes is extensive and includes P450 SCC (Richards *et al.*, 1987), P450 3β-HSD (Magoffin and Erickson, 1994), P450 17α-HYD (Magoffin and Erickson, 1994), P450 ARO (Hickey *et al.*, 1988; Steinkampf *et al.*, 1988), LH receptors (Segaloff *et al.*, 1990), IGF-I (Hernandez *et al.*, 1990), IGFBPs (Hernandez *et al.*, 1991) and inhibin (Zhiwen *et al.*, 1987). The list of LH responsive genes is less extensive compared to FSH. LH positively regulates P450 17α-HYD (Erickson *et al.*, 1985) and IGF-I receptors in theca cells (Bergh *et al.*, 1991).

Strong evidence that IGF-I and gonadotropins act synergistically to control follicle cytodifferentiation has been reported. In general, IGF-I itself has either little or no effect on differentiated function of either granulosa or theca cells. However, when gonadotropins are present, activities of theca and granulosa cells are dramatically enhanced by IGF-I. IGF-I and FSH act synergistically to control progestin and aromatase steroidogenesis (Adashi *et al.*, 1985ab) and LH receptors (Adashi *et al.*, 1985c) whereas IGF-I and LH act synergistically to control androgen biosynthesis (Erickson *et al.*, 1985). In addition, several studies have reported that inhibin locally produced by the DF is able to promote LH dependent synthesis of androgen (Hsueh *et al.*, 1987; Hillier *et al.*, 1991a).

Conversely, high concentrations of E_2, on the order of those found in dominant follicles, have been postulated to cause a selective inhibition of P450 17α-HYD. Magoffin and Erickson (1982) demonstrated that E_2 may act as a competitive inhibitor of P450 17α-HYD activity in the short term and affect gene expression in the long term. Taken together, experimental evidence strongly supports the role of gonadotropins to stimulate transcription and translation of P450 SCC, P450 3β-HSD and P450 17α-HYD required for A_4 biosynthesis. Furthermore, stimulation of P450 ARO in granulosa cells by a similar mechanism permits, conversion of androgens to estradiol; gene expression of the four steroidogenic enzymes is further potentiated by autocrine actions of inhibin and IGF-I.

Regulation of Steroid Hormones Gene Expression at the Ovarian Level

The regulation of expression of the genes encoding P450 SCC and P450 17α-HYD enzymes in bovine theca and luteal cells has been thoroughly studied by Simpson *et al.* (1991, 1992). Chimeric constructs containing fragments of the 5'-end of the bovine P450 SCC and P450 17α-HYD genes were fused upstream of the β-globin or the chloramphenicol acetyltransferase (CAT) gene and subsequently transfected into bovine theca and bovine luteal cells by electroporation. From a series of P450 SCC constructs transfected to bovine luteal cells, it was observed that one construct in particular, which had a deletion from -186 to -100, had a dramatic reduction in forskolin stimulated activity. This indicated that the -186 to -100 region contained a cAMP-responsive element. However, to date no classical cAMP-responsive element with a TGACGTCA sequence has been described in the 5' end of the ovarian steroidogenic genes. Conversely, when the

P450 17α-HYD constructs were transfected in bovine luteal cells, no expression of the CAT or the β-globin gene was observed in the presence or absence of forskolin. These results were consistent with the endogenous expression of the enzyme. Interestingly, when P450 SCC (-186 to +12 bp) and P450 17α-HYD (-297 to +19 bp) constructs were transfected into bovine theca cells in culture, both genes were expressed and were additionally stimulated by forskolin in agreement with the endogenous expression pattern. The authors concluded that failure of the luteal cells to express constructs that contained the 5'-end of P450 17α-HYD gene strongly suggested that during luteinization either a positive transcription factor required for expression of P450 17α-HYD was not present or that a new negative transcription factor was inhibiting the expression of P450 17α-HYD.

In summary, endocrine, cellular and molecular aspects of follicular steroidogenesis have been reviewed. It is clear that studies which address expression of these enzymes and the production of the corresponding steroids during the transition from dominance to atresia are lacking.

Inhibin/Activin

The concept that proteins in addition to steroids hormones of gonadal origin could play a role in the inhibition of gonadotropin secretion from the *par distalis* of the pituitary was proposed 72 years ago (Mottram and Cramer, 1923). A series of experiments performed through the next 10 yr showed that male castration induced development of highly vacuolated, signet-shaped basophil cells in the pituitary. Appearance of these "castration cells" demonstrated a reciprocal relationship between pituitary and gonads suggesting that secretion(s) from the testes had inhibitory effects on pituitary function (reviewed by Ying, 1988). Furthermore, injection of aqueous extracts of testes into castrated male rats, prevented appearance of castration cells and hypertrophy of the pituitary but had no effect on atrophy of accessory sex organs after castration (McCullagh, 1932). McCullagh (1932) named this water-soluble principal from testes extracts "inhibin" because it was capable of preventing pituitary hypertrophy in male rats after castration in contrast to the fat soluble extract responsible for maintaining accessory glands and years later known as testosterone. At the same time, FSH and LH were discovered by Fevold *et al.* (1931). However, direct evidence for the presence of a substance originating in the Sertoli cells of the seminiferous tubules that was responsible for selective inhibition of FSH secretion was not discovered until 1942 by Klinefelter *et al.* (1942). At that time, Klinefelter *et al.* (1942) were studying a syndrome in human males (Klinefelter Syndrome) that was characterized by elevated levels of FSH in urine, oligozoospermia or azoospermia, and near absence of Sertoli cells. These clinical observations demonstrated that absence of a substance produced by the seminiferous tubules resulted in an increase in FSH levels thus suggesting the regulation of FSH secretion by feedback of this gonadal hormone named "inhibin".

Characterization of Inhibin Molecules in Follicular Fluid

Forty-three years later, bovine and porcine inhibin was isolated from follicular fluid by four different laboratories working independently (Miyamoto *et al.*, 1985, 1986 [bovine and porcine]; Ling *et al.*, 1985 [porcine]; Rivier *et al.*, 1985 [porcine]; Robertson *et al.*, 1985, 1986 [bovine]). Inhibin is a member of the TGFβ superfamily which also includes

TGFβ itself, activin, and Muellerian Inhibiting Substance (Findlay, 1993). They are all dimeric molecules comprised of disulfide-bonded homologous polypeptide chains denoted α and β, with only the dimeric form of the protein biologically active. All three combinations (i.e. $\alpha\alpha$, $\alpha\beta$, $\beta\beta$) are typically present in cells (Hillier, 1994; Magoffin and Erickson, 1994). The mature form of inhibin is a heterodimeric glycoprotein composed of a common α-subunit and one of two β-subunits, β_A- (inhibin-A) and β_B- (inhibin-B) whereas the mature form of activin is a homodimeric protein composed of two inhibin β-subunits: activin-A (β_A, β_A), activin-AB (β_A, β_B) and activin-B (β_B, β_B). Activin and TGFβ bind to their own receptors and activate intracellular phosphorylation through a serine/threonine kinase signal transduction mechanism (Park-Sarge and Mayo, 1994). The direct binding of inhibin to rat granulosa cells was first reported by Woodruff *et al.* (1990). In this study, the number of inhibin-A and activin-A receptors in granulosa cells was measured by utilizing a combination of FITC labeling of inhibin-A or activin-A and FACS. The method using FITC-conjugated inhibin overcame the limitation that traditional iodination methods had which resulted in a loss of the binding capacity of inhibin to its own receptor (Woodruff *et al.*, 1990). The authors concluded that rat granulosa cells have approximately 6.4 x 10^3 inhibin-A receptors and 2.3 x 10^4 activin-A receptors/cell. However, to date, the inhibin receptor has not been cloned and/or sequenced and no signal transduction mechanisms described.

Postranscriptional Processesing of Inhibin Forms in Follicular Fluid

In the rat, inhibin precursors are proteolytically processed within the granulosa cells and a 30 kDa mature form is secreted into FF (Bicsak *et al.*, 1988). Conversely, in cattle, inhibin is secreted into FF as an unprocessed dimerized precursor of α (49 kDa) and $\beta_{A,B}$ (58 kDa) subunits (Sugino *et al.*, 1992). The 105 kDa forms first secreted into FF are then post-translationaly processed sequentially to the smaller 95 and 58 kDa dimeric forms, to produce the mature 31 kDa form (Figure 2-3; Sugino *et al.*, 1992). The extracellular processing of the pro-α_N-α_C subunit gives rise to a 6 kDa pro sequence and to a 24 kDa α_N peptide (1-166) and a 20 kDa α_C peptide (167-300; Robertson *et al.*, 1989). Processing of the pre-pro-$\beta_{A,\ B}$ subunit yields a pro peptide of 43 kDa and a 15 kDa $\beta_{A,\ B}$ subunit (Robertson *et al.*, 1992). The 58 kDa inhibin is formed of a α_N-α_C subunit (43 kDa) joined by disulfide bonds to a $\beta_{A,\ B}$ subunit (15 kDa) whereas the 31 kDa mature inhibin is comprised of a α_C subunit (20 kDa) joined by disulfide bonds to a $\beta_{A,\ B}$ subunit (15 kDa; Robertson *et al.*, 1985; Forage *et al.*, 1986; Robertson *et al.*, 1989; Robertson *et al.*, 1992). In the past, several molecular weight forms of dimeric inhibin (116, Knight *et al.*,1989; 120 kDa, Miyamoto *et al.*,1986; 105 kDa Sugino *et al.*,1989; 58 kDa, 31 kDa; Robertson *et al.*,1986, 1989) as well as monomeric α-inhibin precursor proteins (Robertson *et al.*,1989), and large amounts of monomeric α-inhibin (Knight *et al.*,1989; Robertson *et al.*,1989) and monomeric β_A-Inhibin (Robertson *et al.*,1992) have been isolated from bovine FF.

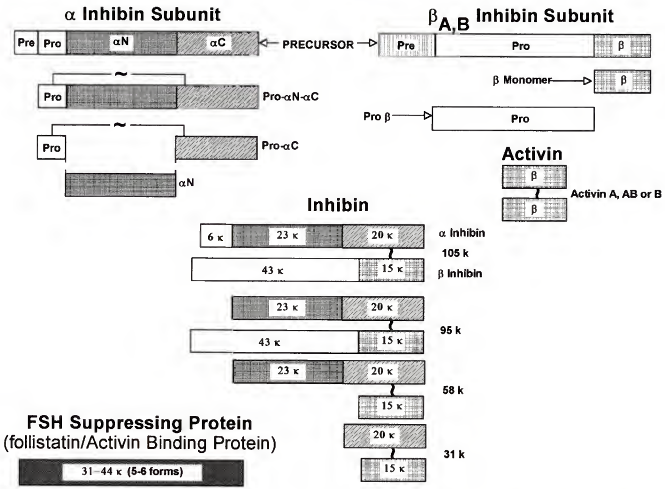

Figure 2-3. Squematic representation of postranslational processing of inhibin/activin and follistatin molecules in bovine follicular fluid.

Endocrine, Paracrine and Autocrine Roles of Inhibin

Similar to other members of the TGFβ superfamily, only dimeric forms of inhibin are biologically active. Hence, FSH secretion from the pituitary is only suppressed by all dimeric forms of inhibin (Miyamoto *et al.*,1986; Robertson *et al.*,1989; Sugino *et al.*,1989), and not by free monomeric α-inhibin (Knight *et al.*, 1989; Robertson *et al.*, 1989). Although these α-inhibin precursor proteins (Robertson *et al.*,1989) and monomeric α-inhibin (Knight *et al.*,1989) do not exert any endocrine feedback at the pituitary level, recent observations suggest that they may have paracrine and autocrine roles at the ovarian level (Findlay *et al.*, 1989; Russell *et al.*, 1994; Schneyer *et al.*, 1991). This view is supported by the observation that a large monomeric α-subunit inhibin precursor (57 kDa) regulates FSH receptor binding and bioactivity (Schneyer *et al.*, 1991) and has an autocrine or paracrine role in regulating fertility (Findlay *et al.*, 1989; Russell *et al.*, 1994). Immunization of ewes against the α_N (1-166, ewe; Findlay *et al.*, 1989; Russell *et al.*, 1994) or the α_C (heifers [1-26; Scanlon *et al.*, 1993] [18-30, 63-72, 101-122; Morris *et al.*, 1993]) peptides of α-inhibin subunit respond quite differently in terms of FSH and inhibin concentrations in plasma, ovulatory mechanism, ovulation rate, pregnancy rate and CL formation. Ewes immunized against α_N (1-166; Findlay *et al.*, 1989; Russell *et al.*, 1994) had decreased inhibin concentrations and increased plasma FSH concentrations as a consequence of reduced negative feedback of inhibin on FSH secretion at the pituitary level. The authors hypothesized that this reduction in negative feedback was due to the binding of antibodies to the α_N portion of the 58 kDa or to larger molecular mass circulating forms of inhibin causing either direct immunoneutralization of these forms or a block in cleavage of the α_N subunit by serum proteases to form the 32 kDa form. Furthermore, even though circulating P_4 concentrations and LH secretion (i.e. basal LH, pulse amplitude and frequency and preovulatory surge) were not affected by immunization, fewer oocytes were present in the oviduct 2 days after ovulation, with either no change or an increase in number of CL-like structures on the ovary (Russell *et al.,*1994). Interestingly enough, only 50% of the ewes conceived in contrast to the control group where 100% were pregnant (Findlay *et al.,* 1989). Collectively, these data suggest that immunization against the α_N peptide impairs fertility via intraovarian mechanisms leading to failure of oocyte release from the follicle. Conversely, immunization of heifers against the α_C peptide of inhibin induced changes in follicular dynamics and increased ovulation rate in heifers (Morris *et al.,* 1993; Scanlon *et al.,* 1993). Moreover, Forage *et al.* (1987) and Wrathall *et al.* (1992) reported increases in FSH concentrations and number of CL, with no impairment in follicular development and CL function, with immunizations of ewes against α_C compared to immunizations against α_N peptide that impaired ovulation and CL development (Russell *et al.,* 1994). The α-inhibin subunit (58 kDa) has been identified *in vitro* as a putative competitor of FSH for FSH receptor binding (Sato *et al.,* 1982; Schneyer *et al.,* 1991). Therefore the increase in FSH concentrations and ovulation rate observed in animals immunized against the α_C peptide can be attributed to immunoneutralization of inhibin and to increased FSH receptor binding at the ovary, thus preventing follicular atresia and increased ovulation rate (Morris *et al.,* 1993).

Collectively, these results clearly support the traditional endocrine role of inhibins in regulation of FSH secretion and in folliculogenesis, but also support important autocrine and paracrine roles of α-inhibin subunits at the ovarian level.

Radioimmunoassay and Immunoradiometric Assay of Inhibin in Follicular Fluid

To date, inhibin concentrations in FF and plasma can be measured either by radioimmunoassay (RIA; Knight *et al.*, 1991; Martin *et al.*, 1991; McLachlan *et al.*, 1986) or by immunoradiometric assay (IRMA; Knight *et al.*, 1991). Inhibin concentrations measured by RIA ($_{RIA}$INH; inhibin $\alpha_c^{1\text{-}26}$gly.tyr [standard and tracer]; rabbit antiserum against $\alpha_c^{1\text{-}32}$ pig inhibin [$\alpha_c^{1\text{-}32}$ pINH] coupled to BSA; Martin *et al.*, 1991) represent total immunoreactivity of inhibin α-subunits and $\alpha\beta$ dimers in plasma or FF. Concentrations of $_{RIA}$INH in FF were correlated positively with EAR ratio, were higher during the growing phase than during the regressing phase of development of the first wave DF and (Guilbault *et al.*, 1993; Ireland and Ireland, 1994), and DF had higher concentrations of $_{RIA}$INH compared to SF (Martin *et al.*, 1991). Furthermore, a parallel increase in FF concentrations of $_{RIA}$INH has been reported with increases in follicle size and E_2 concentrations in the FF (Martin *et al.*, 1991). Conversely, dimeric inhibin concentrations measured by IRMA (sheep antibody against $\alpha_c^{1\text{-}32}$; murine antibody against $\beta_A^{82\text{-}114}$; donkey anti-sheep capture antibody) were negatively correlated with E_2:A_4 ratios and were higher in the regressing DF compared to the growing DF (Guilbault *et al.*, 1993; Ireland and Ireland, 1994).

Characterization of Different Forms of Inhibin in Follicular Fluid: Immunoblot Analysis

Immunoblot analysis has been utilized to characterize changes in the array of different inhibin molecules produced by postranslational processing of the original unprocessed form secreted in FF (Ireland and Ireland, 1994; Miyamoto *et al.*, 1986; Schneyer *et al.*, 1991; Sugino *et al.*, 1992). Schneyer *et al.* (1991) using a rabbit antiserum against human Inhibin $\alpha_c^{1\text{-}25}$gly.tyr detected a 57, 44, 42, 27 and 20 kDa forms thought to be products of postranslational processing of α-Inhibin in human FF. Similar immunoblot results have been reported by Miyamoto *et al.* (1986) and Sugino *et al.* (1992) in bovine FF. Using an antibody specific to the α-inhibin subunit, Miyamoto *et al.* (1986) identified seven forms of inhibin (120, 108, 88 kDa [trimeric=dimeric +monomeric 62 kDa]; 65, 55, and 32 kDa [dimeric]; 62 kDa [monomeric]) in FF. Sugino *et al.* (1992) using the same antibody to the α-subunit and a separate one to β_A-subunit identified seven (105, 95, 55 and 32 kDa [dimeric]; 50 and 40 kDa [αmonomeric]; 55 kDa [$\beta_A$monomeric]) forms of inhibin in FF. More recently, Ireland and Ireland (1994) studied alterations in the amounts of different forms of inhibin with regard to atresia in follicles obtained from the abattoir. Qualitative immunoblot analysis of inhibin of non-reduced FF using mink antiserum against inhibin $\alpha_c^{1\text{-}26}$gly.tyr identified at least eight forms of inhibin in bovine FF containing $\alpha_C^{1\text{-}26}$ epitope (i.e. α-subunits or the α-β dimers; >160, 122, 77, 58, 49, 48, 34 and 29 kDa). Furthermore, the predominant form found in FF was the form having a $M_r \geq$ 122 kDa, and amounts of most forms of inhibin decreased with atresia, with the exception of the 34 kDa form that increased 1.9 -fold.

Similar results were obtained when the proportion of each form relative to the total amount of inhibin in FF were studied. While the proportion of the 34, and ≥ 160 kDa increased during atresia, the proportions of 48, 49 and 122 kDa forms decreased. In addition to immunoblot analysis, Ireland and Ireland (1994) performed RIA and IRMA analyses to determine inhibin concentrations in FF. While inhibin concentrations decreased with atresia as determined by RIA, the concentrations determined by IRMA increased. Hence the authors concluded that neither RIA nor IRMA accurately measured alterations in all forms of inhibin in bovine FF as determined by immunoblot. Collectively, previous studies done in cattle show that inhibin is secreted into FF in large precursor forms that are postranslationaly processed to lower molecular weight forms during follicular atresia. All these studies were performed with follicles obtained from ovaries collected at the abattoir. Therefore, the precise stages of follicle development were not controlled. Furthermore, profiles that document the different changes in inhibin forms present in FF of during the transition from dominance to atresia of first wave dominant follicles and comparison between dominant and subordinate follicles at precise stages of development are lacking.

Inhibin Gene Expression

The three inhibin/activin subunits (α-inhibin subunit, β_A-inhibin subunit, β_B-inhibin subunit) are encoded by separate genes (Esch *et al.*, 1987 [rat]; Forage *et al.*, 1986 [bovine]; Mason *et al.*, 1985 [pig], 1986 [human]). Several studies address the expression of α- and β_A-inhibin in cattle and sheep (Ireland *et al.*, 1994; Juengel *et al.*, 1993;

Rodgers *et al.*, 1989; Torney *et al.*, 1989), rats (Esch *et al.*, 1987; Meurier *et al.*, 1987), pig (Mason *et al.*, 1985) and human (Mason *et al.*, 1986). *In situ* hybridization studies in cattle (Torney *et al.,* 1989) revealed the presence of mRNA for inhibin α-subunit in granulosa cells of antral follicles >0.36 mm in diameter. In addition, hybridization to inhibin α-subunit and β_A-subunits mRNA also was detected in granulosa and theca cells of antral follicles greater than 0.80 mm. Moreover, no hybridization was observed in cells of the CL (Torney *et al.,* 1989). In another study, gene expression of inhibin α and β_A subunits was studied in follicles and corpora lutea obtained from cows and sheeps at the abattoir (Rodgers *et al.*, 1989). Inhibin α and β_A subunit mRNA were detected in antral follicles but neither was detected in ovarian stroma, which potentially could have contained follicles up to 0.5 mm in diameter, or in corpora lutea of cyclic or pregnant cows. In a very recent study, Ireland *et al.* (1994) studied changes in expression of inhibin/activin α, β_A and β_B subunit mRNAs during different stages of differentiation in non-ovulatory follicles in cattle. The follicles in this study were obtained from ovaries collected at a slaughterhouse, which were further classified into non-atretic, atretic and highly atretic based upon the absolute ratio of FF E_2 and P_4 concentrations. The mRNA levels of α, β_A subunits were 2.4- to 5.8-fold higher in non-atretic follicles compared to small follicles and were 4.4- and 9.8-fold lower in highly atretic follicles compared to non-atretic follicles. In addition, changes in amounts of follicular α, β_A and β_B subunit mRNA were positive and highly correlated with intrafollicular concentrations of E_2, ratio of E_2 to P_4, and total immunoreactive inhibin, but were negatively correlated with P_4.

The size of the original α-inhibin bovine cDNA cloned by Forage *et al.* (1986), was about 1.1-1.3 Kb. Juengle *et al.* (1993) reported a single hybridization signal for bovine inhibin α-subunit of 1.8 Kb using a rat cDNA probe whereas Ireland *et al.* (1994) reported a predominant 1.3 Kb band and additional bands at 2.2, 3.3 and 6.4 Kb utilizing a human probe. In another study, Rodgers *et al.* (1989) also reported multiple bands utilizing a bovine probe with the predominant band at 1.7 Kb and a much weaker one at 3.0 Kb. Similar discrepancies with size of the hybridization signal for the β_A-subunit have been described by Ireland *et al.* (1994), Juengle *et al.* (1993) and Rodgers *et al.* (1989). Utilizing a human cDNA probe, Ireland *et al.* (1994) detected a 6.4 Kb band and two less prominent bands at 2.6 and 4.0 Kb whereas Juengle *et al.* (1993) detected a prominent band at 5.7 Kb and a minor band at 4.7 Kb. In contrast to this two previous studies, Rodgers *et al.* (1989) utilizing a bovine cDNA probe detected a single hybridization band at 6.5-6.9 Kb. Collectively, although the changes in expression of inhibin/activin α, β_A and β_B subunit mRNA in bovine follicles have been addressed in various studies, a detailed study that measures qualitative and quantitative changes in gene expression of inhibin subunits at precise stages of follicle development during dominance and atresia of the first wave of follicle development is lacking.

Several studies have been completed to document changes in concentrations and processing of inhibin in FF as well as changes in inhibin subunit mRNA levels in follicle walls, the majority of these studies were performed in follicles from ovaries collected at the abattoir. Thus, the precise physiological status of these follicles with regards to their stage in development was sometimes difficult to determine. Therefore, future research should focus attention on the study of these patterns of inhibin changes utilizing an experimental model that allows for control of follicle development with recovery of dominant and subordinate follicles at critical stages of dominance, atresia, transition of dominance to atresia and sustained dominance.

Insulin-like Growth Factors (IGF) and IGF-Binding Proteins (IGFBPs)

Ovarian physiology requires rapid and continuous regulation of growth associated with folliculogenesis and oogenesis. Granulosa cells provide the cytoarchitectural support for the developing oocyte and participate in follicular antrum formation while theca cells surround granulosa cells and form the exterior wall to the follicle. Both cell types undergo extensive proliferation and functional differentiation to develop from preantral to antral follicles, and once the follicle is selected and becomes dominant, further growth is required to achieve ovulatory size. In human and in most large domestic species, follicle size increases from millimeters to centimeters during this period of time. Conversely, the cohort of follicles that does not became selected and/or the non-ovulatory dominant follicle become atretic and cell growth is arrested. Therefore, the regulation of cell proliferation in ovarian follicles requires both stimulatory and inhibitory mechanisms. Follicular development is primarily regulated by LH and FSH (Richards, 1980; Ireland and Roche, 1987, Lucy *et al.*, 1992), but ample evidence is emerging that growth factors which are themselves products of gonadotropin action, may act locally on follicle cells to either amplify or attenuate the timing and direction of granulosa and theca cell differentiation (Erickson *et al.*, 1994; Hillier *et al.*, 1994).

Characterization of IGF-I and IGF-II Molecules

The IGF-I and II molecules were first purified from human plasma and sequenced by Rinderknecht and Humbel 17 yr ago (Rinderknecht and Humbel, 1978ab). Both IGF are low molecular weight single chain polypeptides that are structurally related to proinsulin and promote cellular mitosis and differentiation in a variety of tissues and systems (Giudice, 1992). Furthermore, IFG-I is formed of 70 amino acids whereas IGF-II is formed by 67 amino acids. Both IGF have 40% structural homology with insulin and 60% of structural homology with each other (Giudice, 1992). Insulin-like growth factor-I is produced by granulosa cells under control of FSH and E_2 (Hammond *et al.*, 1985) and is shown to have a synergistic effect with FSH to stimulate granulosa cells *in vitro* (Adashi *et al.*, 1991; Giudice, 1992; Hammond *et al.*, 1991). Detailed studies of the cellular sites for gene expression of IGF-I and IGF-II have revealed that IGF-I is localized in granulosa (Hernandez *et al.*, 1989; Oliver *et al.*, 1989) and IGF-II is localized in theca cells (Hernandez *et al.*, 1989) of rat ovaries. Furthermore, expression of the IGF-I gene has been identified in granulosa cells of cattle (Echternkamp *et al.*, 1991; Spicer *et al.*, 1993) and pigs (Hammond *et al.*, 1985).

Characterization of IGF-I and IGF-II Receptors

The IGF exert their physiological actions by interacting with specific cell surface membrane receptors, described as type I and type II receptors. The type I receptor referred as the IGF-I receptor, is tetrameric in nature (2α and 2 β subunits), has low affinity for insulin, has tyrosine kinase activity and preferentially binds IGF-I. On the other hand, the type II receptor is monomeric in nature, does not have any affinity for insulin, does not have tyrosine kinase activity, prefentially binds IGF-II and is therefore called the IGF-II receptor (Giudice, 1992). Receptors for IGF-I have been localized in rat (Adashi *et al.*, 1988; Davoren *et al.*, 1986) and porcine (Baranao and Hammond, 1984) granulosa cells and rat theca cells (Hernandez *et al.*, 1991). A similar pattern of expression was observed for the IGF-II receptor in rat ovaries (Hernandez *et al.*, 1991). Experimental evidence indicates that expression of IGF receptors is under gonadotropin control because hypophysectomy decreases the biosynthesis of IGF receptors, and treatment with FSH and LH restores IGF receptors to levels similar to those prior to hypophysectomy (Davoren *et al.*, 1986; Hernandez *et al.*, 1991). The IGF-I binding to its receptor promotes dimerization and transphosphorylation, which in turn promotes phosphorylation of cellular substrates (reviewed by Park-Sarge and Mayo; 1994). Some of the characterized substrates for tyrosine kinase phosphorylation include phosphatidylinositol kinase, phosphatidylinositol-specific phospholipase C-γ, phospholipase C-II, Raf-1 and ras GTPase activating protein (reviewed by Park-Sarge and Mayo; 1994).

Concentrations of IGF-I and IGF-II in Follicular Fluid

Changes in plasma and FF concentration of IGF-I in cattle have been reported in several studies (Badinga *et al.*, 1992; Echternkamp *et al.*, 1994; Spicer and Enright, 1991; Stanko *et al.*,1994). Echternkamp *et al.* (1990) reported that IGF-I concentrations did not differ in serum and plasma collected simultaneously from the same animal. However, the relative contributions of absorption from serum vs local secretion by granulosa cells of ovarian follicles to concentrations of IGF-I measured in bovine FF are unknown (Echternkamp *et al.*,1990). Whereas Badinga *et al.* (1992) reported no changes in FF concentrations of IGF-I in first wave dominant follicles collected on days 5, 8 and 12 of the estrous cycle, Echternkamp *et al.* (1994) reported that FF IGF-I concentrations increased with increasing concentrations of E_2 in FF in periovulatory follicles.

Biological effects of IGF-I and IGF-II at the ovarian level

Considerable effort has been devoted in the last 10 yr to identify the biological effects of IGF-I in follicle physiology. IGF-I alone elicits a dose dependent increase in inhibin synthesis (Zhiwen *et al.*, 1987) but otherwise IGF-I itself appears to have little or no effect on differentiated functions of granulosa cells. On the other hand, IGF-I has been shown to act synergistically with FSH and LH to stimulate: aromatase activity of granulosa cells in rats (Adashi *et al.*, 1985a,b), humans (Berg *et al.*,1991; Erickson *et al.*,1989,1990; Christman *et al.*, 1991) and pigs (Veldhuis *et al.*,1985,1986,1987); P450 aromatase mRNA expression in human granulosa cells (Steinkamp *et al.*, 1 988); P450 side chain cleavage mRNA expression in rat theca cells (Magoffin *et al.*, 1990) and porcine granulosa cells (Urban *et al.*,1990); LH receptor synthesis in rat granulosa cells (Adashi *et al.*,1985c); inhibin secretion in rat granulosa cells (Bicsak *et al.*, 1986), and androgen biosynthesis in rat theca cells (Hernandez *et al.*, 1988).

IGF-binding Proteins in Follicular Fluid

A dominant follicle evolves according to a defined sequence and temporal pattern of gene expression. Traditionally, FSH and LH have been described as playing the leading role in this process. More recently, evidence has become available to indicate that IGF themselves are the products of gonadotropin action and have a very active role in folliculogenesis by acting locally to modulate the timing and direction of granulosa and theca cell differentiation and gene expression. Furthermore, an increasing body of evidence suggests that in the ovary, the biological responses of IGF-I and IGF-II under normal FSH stimulation also may be regulated by IGFBPs (Ui *et al.*, 1989). These IGFBPs may bind to IGF-I and IGF-II present in the FF and therefore selectively negate their tropic activity (Ui *et al.*, 1989; Magoffin and Erickson, 1994). The IGFBPs are a complex family of proteins that bind IGF-I and IGF-II in body fluids and tissues. The major functions of IGFBPs are: reservoir of IGF molecules, transport of IGF to target tissues, increasing the half-life of IGF, regulating endocrine effects of IGF in peripheral circulation, and modulating paracrine and autocrine effects of IGF in specific tissues (Giudice, 1992).

The discovery of follicular fluid IGFBPs originated serendipitously from the search of porcine FF for an endogenous inhibitor of FSH stimulated E_2 production in a well characterized rat granulosa cells assay (Ui *et al.*, 1989; reviewed by Erickson *et al.*, 1994). The amino acid sequence analysis of the amino-terminal segment of the newly purified protein was quite similar to the amino acid sequence of a 53 kDa growth-hormone dependent IGFBPs isolated from human serum and later designated as IGFBP-3. The molecular cloning of the inhibitor from a porcine cDNA library and the deduced amino acid sequence of the isolated cDNA confirmed that the inhibitor was indeed the porcine homolog of the human IGFBP-3. The incorporation of affinity chromatography into the procedure utilized to purify the FSH-inhibitor permitted the isolation of IGFBP-2 and -3 as well as three novel IGFBPs which were later designated IGFBP-4, -5 and -6 (reviewed by Erickson *et al.*, 1994). Using ligand blot analysis, immunoprecipitation, *in situ* hybridization and Northern blot analysis, six IGFBPs (hIGFBP-1 [28 kDa], -2 [34 kDa], -3 [43&40 kDa], -4 [30&24 kDa], -5 31-29 kDa], and -6 [22 kDa]) have been identified in the human ovary (Giudice, 1992; Zhou and Bondy, 1994), five IGFBPs (rIGFBP-2 [30.5 kDa], -3 [45&40 kDa], -4 [28&24 kDa], -5 [30&29 kDa], and -6 [26.5 kDa]) in the rat ovary (Guidice, 1992; Liu *et al.*, 1993; Nakatani *et al.*, 1991), four IGFBPs (pIGFBP-2 [34 kDa], -3 [44&40 kDa], -4 [22 kDa] and -5 [30 kDa]) in the pig ovary (Guidice, 1992; Grimes *et al.*, 1994; Shimasaki *et al.*, 1991) and two IGFBPs (bIGFBP-2 [34 kDa] and -3 [40&44 kDa]) in the bovine ovary (Echternkamp *et al.*, 1994). In addition, 29-27 and 22 kDa bands were detected but not identified by immunoprecipitation in bovine FF (Echternkamp *et al.*, 1994).

At least three IGFBPs (2, 4 and 5) have been shown to have antigonadotropic effects on ovarian FSH stimulation in humans, rats, pigs and cows. In humans, IGFBP-2 (Cataldo and Guidice, 1992; San Roman and Magoffin, 1993) and IGFBP-4 (Guidice *et al.*, 1993) levels increase in atretic follicles whereas IGFBP-3 levels in dominant and atretic follicles decrease during folliculogenesis (San Roman and Magoffin, 1993). In rats, IGFBP-4 and 5 mRNAs levels are increased in granulosa cells of atretic follicles but are not expressed or are expressed at very low levels in granulosa cells of dominant follicles (Erickson *et al.,*1992a,b). In pigs, IGFBP-2 (Howard and Ford, 1992), IGFBP-4 and 5 (Grimes *et al.,* 1994) are present predominantly in atretic and in small immature follicles. Similar results have been described in cattle, where FF IGFBP-2, and amounts of two unidentified IGFBPs forms (29-27 kDa and a 22 kDa) were related inversely to FF E_2 concentrations (Echternkamp *et al.,*1994). There is at least one report in cattle that provides evidence to indicate that IGFBPs levels were higher in FF from bovine follicles that contained oocytes with reduced developmental potential and in follicles that contained oocyte cumulus complexes that failed to develop to the blastocyst stage (Hazeleger *et al.,* 1993). Collectively, ligand blot analysis of FF from human and rat follicles indicate that IGFBP-3 is the most predominant IGFBPs in FF and that amounts IGFBP-2, -4 and -5 are higher in atretic follicles. In addition, studies in porcine preovulatory follicles indicate that low amounts of IGFBP-2, -4 and -5 are present in these follicles, and that IGFBP-3 is the most abundant form present in FF. In cattle, one study partially characterized the presence of IGFBPs in FF during the periovulatory period but did not include atretic dominant follicles. Furthermore, although several studies comparing IGFBPs contents in dominant and atretic follicles of rat pig and humans are available, similar studies in cattle FF are lacking.

Mechanisms that Regulate the Amounts of IGFBPs in Follicular Fluid

There are at least two FSH dependent mechanisms that are thought to regulate the FF amounts of IGFBP-2, -4 and -5 in rat ovarian follicles (Liu *et al.*, 1993). The first potential mechanism to regulate FF amounts of IGFBPs involves inhibition of gene expression of IGFBP-2, -4 and -5. The FSH signal transduction mechanism may act either to inhibit gene transcription or to increase degradation of mRNAs for these low M_r IGFBPs (Liu *et al.*, 1993). Several *in vitro* studies support this view (Adashi *et al.*, 1990,1991; Liu *et al.*, 1993). These studies show that treatment of granulosa cell cultures with FSH produces a dramatic decrease in the levels of mRNA for IGFBP-4 and -5 compared to control cultures. The second potential mechanism to regulate FF amounts of IGFBPs involves stimulation of protease(s) that cleaves IGFBP-2, -4 and -5 into lower molecular mass fragments that do not bind IGF in FF (Liu *et al.*, 1993). Several lines of evidence support this concept. *In vitro* studies done in rat granulosa cell cultures clearly show that FSH stimulates the production of proteases that degrade IGFBP-4 and -5 in culture medium (Liu *et al.*, 1993). In these experiments, treatment of granulosa cell cultures with FSH produced the appearance of lower M_r band detected by immunostaining (IGFBP-4: 21-5 and 17.5 kDa [derived from the 28 and 24 kDa bands]; IGFBP-5: 21 kDa [derived from the 29 kDa band]) but not by ligand blot analysis which suggested that the new fragments formed were not able to bind IGF-I. Furthermore, the M_r of these newly formed bands by proteolysis of the native forms of IGFBPs suggest that additional 6.5 and 8 kDa fragments may have been produced but not detected by immunostaining or ligand blot in order to add up to the M_r of the native IGFBPs. A similar specific protease has been described in serum of pregnant rats (Davenport *et al.*, 1990, 1992) or porcine FF (Grimes and Hammond, 1994) which degrades IGFBP-3 into fragments that do not bind

IGF-I and IGF-II with high affinity (Davenport *et al.*, 1990, 1992). Additional support for both FSH dependent mechanisms comes from the observation that FF concentrations of FSH are much higher in non-atretic DF compared to atretic follicles (McNatty *et al.*, 1975).

Although the main role of IGFBPs is to bind free IGF in FF, and therefore selectively negate IGF trophic activity, new evidence also supports another potential mechanism whereby low M_r IGFBPs would act at the follicular level as inhibitors of cell proliferation (Bicsak *et al.*, 1990). Support for this idea comes from *in vitro* studies with rat granulosa cell cultures where IGFBPs inhibited FSH stimulated [^{3}H] thymidine uptake and from having ruled out the actions IGFBPs at the FSH receptor (Bicsak *et al.*, 1990). Although demonstration of a direct interaction of IGFBP-2 or 3 with granulosa cells through cell surface receptors has not been described, the presence of a fibronectin like binding motif (Arg-Gly-Asp) in IGFBP-2 (Baxter and Martin, 1989) and the binding of IGFBP-1 to fibronectin and laminin (Jones *et al.*, 1993) gives further support to the idea that IGFBPs may function as general inhibitors of cell proliferation through direct interactions with granulosa cell receptors. In summary, non-atretic dominant follicles may be able to reduce the amounts of low M_r IGFBPs in FF through the inhibition of gene expression or through the induction of proteases that would cleave these binding proteins into fragments not able to bind IGF in FF, and would allow IGF-I and IGF-II to exert their stimulatory actions on follicle cells.

Ovarian Gene Expression of IGFBPs

Two very detailed studies of the cellular sites of synthesis of the IGFBPs in the rat adult ovary have been done utilizing *in situ* hybridization techniques (Nakatani *et al.*,1991; Erickson *et al.*, 1992ab). These studies have provided enough evidence to support the view that at least in the rat, only IGFBP-2, -4 and -5 are locally produced in theca or granulosa cells of follicles. Furthermore, whereas IGFBP-6 was shown to be in theca externa, smooth muscle and stroma cells, no signs of mRNA production for IGFBP-1 and -3 was detected in ovarian tissues with the exception of IGFBP-3 in the CL. IGFBP-2 mRNA localization was restricted to theca interna cells of secondary, tertiary, dominant, preovulatory and atretic follicles whereas IGFBP-4 and -5 mRNA was localized in granulosa cells of atretic preantral and antral follicles and in theca interna of atretic follicles. Gene expression studies in the pig ovary have shown similar pattern (Grimes *et al.*, 1994; Samara *et al.*, 1992). Messenger RNA for IGFBP-2 was most abundant in granulosa cells, lower in theca cells, and lowest in luteal cells. No IGFBP-3 mRNA could be detected in granulosa cells, and luteal cells expressed 15- to 65-fold greater levels than thecal cells (Samara *et al.*, 1992). Furthermore, Grimes *et al.* (1994) studied gene expression of IGFBP-1, -4, -5 and -6 in pig granulosa cell cultures to determine the effects of FSH and IGF-I stimulation. Results from these experiments indicate that mRNAs for IGFBP-1 and -6 are not expressed in granulosa cells of pig follicles, and that mRNAs for IGFBP-4 and -5 are expressed in these cells with production attenuated by FSH and stimulated by IGF-I treatment. The mRNA distribution of the IGFBPs in the human ovary was thoroughly studied by Zhou and Bondy (1993). In this study, IGFBP-1 mRNA was not detected, but IGFBP -2 to -5 showed an unique ovarian distribution. IGFBP-3 mRNA was detected only in endothelium of ovarian blood vessels, IGFBP-2 was abundant in granulosa and theca cells of ovarian follicles, IGFBP-4 was localized in granulosa cells of follicles and in endothelium of blood vessels, and IGFBP-5 was expressed in granulosa cells of all follicles studied (Zhou and Bondy; 1993). In cattle, although various studies (Echternkamp *et al.,* 1994; Stanko *et al.,* 1994) have been done to identify the different IGFBPs present in follicular fluid, mRNA studies to quantitate gene expression of IGFBPs are lacking. Collectively, results from mRNA studies from human, rat and pig follicles indicate that IGFBP-2, -4 and -5 are expressed in follicle tissues, IGFBP-6 in stromal tissue and IGFBP-1 and IGFBP-3 are not expressed in follicle tissue. However, IGFBP-3 is expressed in CL tissue. Furthermore, a review of published literature indicates that mRNAs studies of IGFBPs in cattle are lacking.

In summary, IGF-I and II and IGFBPs are produced in ovarian follicles throughout folliculogenesis but conflicting reports are unclear as to whether concentrations of IGF-I are elevated or not in dominant follicles when compared to subordinate follicles. IGF-I plays an important role in folliculogenesis by synergizing with FSH in stimulating granulosa cells to proliferate and differentiate, but the presence of IGFBPs in FF suggests a pivotal role of these molecules in binding to FF IGF and therefore suppressing the effects of IGF-I and II at the autocrine and paracrine level. Several studies in the past have described changes in IGF-I and II and IGFBPs during the follicular phase of the estrus/menstrual cycle; however, a detailed study in cattle that compares IGF-I and II and

IGFBPs changes in dominant vs subordinate follicles as well as in E_2 active versus atretic dominant follicles is lacking.

CHAPTER 3

PROGESTERONE CONCENTRATION AND mRNA LEVELS FOR P450 CHOLESTEROL SIDE-CHAIN CLEAVAGE AND P450 3β-HYDROXYSTEROID DEHYDROGENASE IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES

Introduction

Follicular development in monoovulatory species proceeds through the stages of recruitment, selection and dominance (Goodman and Hodgen, 1983; Ireland and Roche, 1987, Lucy et al., 1992; Fortune, 1994). During the bovine estrous cycle, follicular growth is characterized by development of one or two dominant anovulatory follicles before the terminal preovulatory DF is matured and ovulated. Development of the first DF appears to be the most consistent and predictable event related to follicular dynamics during the bovine estrous cycle (Savio *et al.*, 1993), and indeed turnover and atresia of this follicle can be prevented for a 15 day (d) period by prostaglandin induced luteolysis and insertion of a Norgestomet implant on d8 of the estrous cycle (Savio *et al.*, 1993). Under this hormonal environment, the first wave DF continues to grow at a rate of 0.7 mm/day from d8 to d23 of the estrous cycle and avoiding normal turnover and atresia.

Steroidogenesis in bovine preovulatory follicles requires uptake or *de novo* synthesis of cholesterol and conversion of cholesterol to pregnenolone by P450 SCC (Miller, 1988). Pregnenolone is further metabolized to either 17-hydroxy pregnenolone and DHEA by P450 17α-HYD through the Δ^5 pathway or to P_4 by cytochrome P450 3β-HSD (Fortune, 1986; Fortune and Quirk, 1988). Dehydroepiandrosterone is converted to A_4 by P450 3β-HSD in theca interna cells (Fortune, 1986; Fortune and Quirk, 1988). Subsequently, in granulosa cells, A_4 undergoes aromatization by P450 ARO to E_2 supporting the concept of the "two cells" theory (Falk, 1959; Fortune, 1986; Fortune and Quirk, 1988; Lacroix *et al.*, 1974). In contrast to rat preovulatory follicles where the Δ^4 pathway is the major pathway for A_4 production, in bovine preovulatory follicles most A_4 production occurs via the Δ^5 pathway (Fortune and Quirk, 1988).

Follicular fluid collected from ovarian follicles during the first wave of follicle development (Ireland and Roche, 1983; Martin *et al.*, 1991) has been utilized to determine steroid concentrations (P_4 and E_2) and classify follicles as EI (atretic) or EA (dominant) based upon the PERT (PERT$<$1, EI or atretic; PERT$\geq$1, EA or dominant) in FF, the macroscopic appearance of the surface of the follicles (Grimes and Ireland, 1986), or the comparison of histological and non-histological indices of atresia (Grimes *et al.*, 1987). Furthermore, using absolute FF P_4 concentrations, Ireland and Roche (1983) classified follicles as E_2 inactive (atretic; $P_4=108\pm25$ ng/ml) or E_2 active (non-atretic; $P_4=36\pm5$ ng/ml). More recently, Ireland and Ireland (1994) classified follicles as highly atretic ($P_4=516\pm113$ ng/ml), atretic ($P_4=66\pm48$ ng/ml), and E_2 active ($P_4=20\pm3$ ng/ml). Similar results have been reported by Martin *et al.* (1991). Collectively, these studies indicate that ovarian follicles could be classified as atretic (EI) or non-atretic (EA) based upon FF P_4 concentrations. Furthermore, ovarian follicles with FF P_4 concentrations above 100 ng/ml could be classified as atretic or EI while those with FF P_4 concentrations below 100 ng/ml could be classified as non-atretic or EA.

More recently, daily ultrasonographic observations of bovine ovarian follicles have provided direct evidence of the patterns of follicle development and have permitted the identification of the dominant and the cohort of subordinate follicles in bovine ovaries as early as day 4 of the estrous cycle (de la Sota *et al.,* 1993; Ginther *et al.,* 1989; Lucy *et al.,* 1992; Savio *et al.,* 1988, 1990; Sirois and Fotune, 1988). In fact, a combination of both approaches (i.e. ultrasonography and steroid profiles in FF) has provided the opportunity to describe more accurately the pattern of growth and the endocrine and biochemical differences between first wave DF and SF at days 5, 8 and 12 of the estrous cycle (Badinga *et al.*, 1992). In this study, endocrine and biochemical characteristics of E_2 active DF (D5), transitional E_2 active DF (D8) and atretic DF (D12) were compared to the cohort of SF. Follicular fluid P_4 increased during the process of atresia, and also was higher in SF than in DF. However this study did not address gene expression of the steroidogenic enzymes of these follicles. The levels of mRNA, protein and activities of P450 SCC and P450 3β-HSD in bovine follicles from ovaries obtained at the slaughter house have been reported by Funkenstain *et al.* (1983, 1984), Hinshelwood *et al.* (1993) and Rodgers *et al.* (1986ab, 1987). More recently, follicles obtained from ovaries at precise stages of the estrous cycle were utilized for mRNA studies during the periovulatory period (Voss and Fortune, 1993a) or at different stages of follicle development during the first 10 days of the first wave of follicle development (Xu *et al.*, 1994). The mRNA levels for P450 SCC and P450 3β-HSD of periovulatory DF before and after the LH surge were studied by Voss and Fortune (1993a). P450 SCC and P450 3β-HSD mRNA were localized in granulosa and theca cells of DF before and after the LH surge. It also was reported that although FF P_4 concentrations increased after the LH surge, the mRNA levels for both steroidogenic enzymes did not parallel this increase. It was concluded that increased P_4 within the ovulatory follicle between the LH surge and ovulation may have resulted from a decreased follicular capacity to metabolize progestins to androgens and/or the continued maintenance of activity of both enzymes. Xu *et al.* (1994) used *in situ* hybridization techniques to study gene expression of mRNA for P450 SCC in follicles obtained at 0, 2, 4, 6, 8 and 10 days after initiation of the first wave of follicle development. P450 SCC mRNA was localized in the theca interna of well developed follicles and in granulosa cells of follicles ≥4 mm. However, no description of the steroid concentrations in follicular fluid nor stage of follicle development (i.e. dominant vs subordinate or atretic vs non-atretic) were available to integrate these responses with enzyme gene expression.

In summary, although several studies addressed mRNA levels of P450 SCC and P450 3β-HSD in follicles, a detailed study that compares mRNA levels of these steroidogenic enzymes in DF and SF at defined stages of follicle development and under circumstances of altered follicle development has not been conducted. Our laboratory has developed the capability to obtain DF and SF at defined stages of the first wave of follicle development on a within cow basis and developed the capability of maintaining dominance of the first wave DF. The integration of both techniques into one experimental model provides an unique opportunity to study endocrine and molecular aspects of dominance and atresia in cattle.

The first objective of this chapter was to develop an experimental model to obtain first wave DF and SF at defined stages of development. Establishment of such a model would permit a detailed study of the endocrine and molecular aspects of dominance and atresia in individual follicles. The rational for selecting whole individual follicle (DF or SF) instead of isolated granulosa and theca cells of individual follicles as the experimental unit for the mRNA studies was based on the need to maximize the total RNA yields that could be obtained from SF in order to probe for different messenger RNAs and the large number of follicles expected to be obtained per cow ($n \geq 4$) such that it will not be physically possible to process all follicles at the time of ovariectomy. The DF were collected at four stages of follicular development during the first wave: 1)D5 E_2 active DF; 2) D8 transitional E_2 active DF; 3) D12 atretic DF (D12-I) and (4) D12 E_2 active sustained DF (induced by luteolysis and insertion of a PI on d8 for 4d) Subordinate follicles at these four stages of follicle development also were examined. The rationale for selecting follicles at D5, D8 and D12-I was to characterize follicle function during the transition from E_2 active to atresia of the DF. The rationale for selecting D12+I was that this simple hormonal manipulation applied to the first wave sustained DF and would provide the opportunity to study the endocrine and molecular responses associated with maintenance of follicular dominance and avoidance of atresia.

The second objective of this chapter was to study qualitative and quantitative changes in mRNA levels of P450 SCC and P450 3β-HSD in DF and SF at four defined stages of development. In addition, we wanted to test the hypothesis that increases in FF P_4 production observed during the process of atresia of the DF and SF was paralleled by increases in the levels of P450 SCC and P450 3β-HSD mRNA.

Materials and Methods

Experimental Animals and Reproductive Management

Twenty-four non-lactating, normal cycling Holstein cows were assigned randomly to one of four experimental groups representing different stages of follicular dominance in a split plot design. The experiment consisted of two period (1 and 2; Fig.3-1, A) of synchronized estrous cycles at the end of which the ovary bearing the DF was removed by unilateral ovariectomy per vagina (Drost *et al.*, 1992).

Cows were injected intramuscularly with 25 mg prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; Lutalyse, Upjohn Co., Kalamazoo, MI) to induce luteal regression and estrus (Fig. 3-1, A). At d7 of the subsequent cycle, a PI (6 mg Norgestomet, Synchro-mate B, CEVA Laboratories Inc., Overland Park, KS) was implanted into the ears of all cows and left in place for 9 days (d 7-d 16). At 2 days before removal of the implant (d 14), an injection of $PGF_{2\alpha}$ was administered intramuscularly. To detect behavioral estrus activity, cows were observed for 1 h at 0700, 1300 and 1900 h. Tailheads were painted (Impervo Brand, Benjamin Moore, Montvalo, NJ) and chalked (All-weather Paint Stick, Laco Industries Inc., Chicago, IL) as an aid for estrus detection (Macmillan *et al.*, 1988). Following detected estrus, cows entered into experimental period 1 during which unilateral ovariectomy was performed on the ovary bearing the DF at various experimental stages.

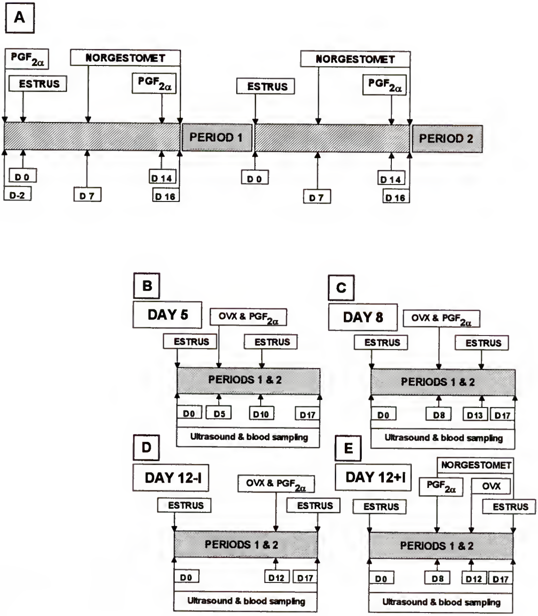

Figure 3-1. Experimental details regarding design, estrus synchronization, ultrasonography, blood sampling and surgery of experimental animals. Top panel (A): The experiment consisted of two periods (1 and 2) of synchronized estrous cycles at the end of which the ovary bearing the DF was removed by unilateral ovariectomy per vagina. Bottom panel. Four experimental groups were included: B) D5 (n=6; E_2 active DF, C) D8 (n=6; non-E_2 active DF), D) D12-I (n=6; atretic DF, without PI inserted and PG injected on d8) and E) D12+I (n=6; active-sustained DF, with PI inserted and PG injected on d8).

Following ovariectomy, cows were injected with $PGF_{2\alpha}$, and on d 7 of the following cycle cows were resynchronized with the same regime as in previous experimental period prior to period 1, and the remaining ovary removed within the same experimental groups during period 2.

The four experimental groups reflect ovariectomies to recover DF at: (1) D5 (n=6; E_2 active DF, Fig. 3.1, B), (2) D8 (n=6; transitional E_2 active DF, Fig. 3.1, C), (3) D12-I (n=6, atretic DF, without PI inserted and $PGF_{2\alpha}$ injected on d 8, Fig. 3.1, D) and (4) D12+I (n=6; E_2 active-sustained DF, PI inserted and PG injected on d 8 and removed on d 12, Fig. 3.1, E).

Ultrasonographic Examination and Blood Collection

Day of estrus was considered d 0 of the estrous cycle. Beginning on the day of the second $PGF_{2\alpha}$ injection (d 14) of both pre-experimental periods (Fig. 3-1, A), cows were examined daily by transrectal ultrasonography utilizing an Equisonics 300A linear-array ultrasound scanner equipped with a 7.5 Mhertz transducer (Tokyo Keiki Co. Ltd., Tokyo, Japan) to determine accurately the day of ovulation (d 1). Ultrasonography then was performed daily for the first 5-12 days of the cycle (during each experimental period [1 and 2]) to follow the pattern of follicular growth and development in both ovaries. Ovarian maps containing the size and relative position of follicles greater than 3 mm and CL were drawn daily (Savio *et al.*, 1988; Sirois and Fortune, 1988; De la Sota *et al.*, 1993). Day of emergence and size of the dominant and cohort subordinate follicles during the first follicular wave of the synchronized experimental estrous cycle were determined by retrospective examination of the ovarian maps and were used to identify the ovary for unilateral ovariectomy (De la Sota *et al.*, 1993). For statistical analyses, follicles were grouped into three classes as described previously (class 1: 3-4 mm, class 2: 5-9 mm and class 3: ≥10 mm) and dominance measured as the daily difference in size between the dominant and the largest subordinate follicle (Lucy *et al.*, 1992; Wolfenson *et al.*, 1994).

Blood samples were collected daily before ultrasonography by jugular venipuncture into evacuated heparinized tubes (Vaccutainer®, Becton Dickenson, East Rutherford, NJ) and placed immediately in ice. Samples were centrifuged at 3000 x g (4°C) for 30 minutes. Plasma was harvested and frozen at -20°C until assayed for P_4.

Tissue Collection and Handling

Transvaginal unilateral ovariectomies (Drost *et al.*, 1992) were performed after the last ultrasonographic examination for each experimental group. After ovariectomy, the ovary was placed in a saline ice bath, and carried to the laboratory adjacent to the surgery room. The DF and the two or three largest SF were dissected from each ovary, measured, weighed, FF collected by aspiration with a syringe, FF volume measured and FF stored at -20°C for further assay of hormones as described below. The follicle walls were weighed, snap frozen in liquid nitrogen and stored at -70°C until used for mRNA extraction as described below.

Assay of Progesterone in Plasma and Follicular Fluid

Progesterone (P_4) was measured in 200 μl aliquots (DF, n=44; SF, n=44) of unextracted diluted FF (0.5 to 3.0 μl of native FF equivalent; Thatcher *et al.*, 1991) and in 1.0 ml aliquots of extracted plasma (Knickerbocker *et al.*, 1986). The intra- and inter-assay coefficients of variation were, respectively, 3.81% and 9.25% for assays in FF and 10.25% and 9.57% for assays in plasma. The minimum detectable concentration of P4 in FF and in plasma samples was 156 pg/ml. Ovarian follicles were further classified as either atretic (EI; $P_4 \geq 100$ ng/ml; ultrasonographic data (size, day of wave, dominant or subordinate) or non-atretic (EA; $P_4 < 100$ ng/ml; ultrasonographic data [size, day of wave, dominant or subordinate]).

RNA isolation

Total RNA was isolated from DF and SF walls by the guanidinium thiocyanate method as modified by Puissant and Houdebine (1990; Appendix 1) and RNA concentrations were estimated from optical absorbance at 260 nm (absorbance unit = 33 μg/ml). When necessary, SF tissue samples (EPR <1; Ireland and Roche, 1982, 1983) were pooled (1-5 samples/pool) to achieve a minimum of 100 μg tissue/pool prior to RNA isolation. The extracted RNA samples were treated with lithium chloride to remove residual polysaccharides (Puissant and Houdebine 1990; Appendix 1).

Northern Blot and Dot Blot Analyses

Northern blot analysis (Thomas, 1980; Appendix 2) was performed on two individual samples (DF) from each experimental treatment (n=8). Ten micrograms of total RNA per sample were separated in a 1.5% agarose gel containing 2.2 M formaldehyde. After electrophoresis, the RNA preparations and a 0.24-9.5 Kb ladder (Gibco BRL, Gaithersburg, MD) used as molecular size markers were visualized by ethidium bromide staining. The quality of the isolated RNA was evaluated by the presence of distinct 18S and 28S ribosomal RNA bands. RNAs were transferred to Bio Trans nylon membranes (0.2 μm, ICN Biomedicals, Inc. Aurora, OH) and each nylon filter was probed sequentially for either P450 SCC mRNA and 28S rRNA or for P450 3β-HSD mRNA and 28S rRNA.

Dot blot analysis was performed as described previously (Simmen *et al.*, 1992; Appendix 3) on individual follicle samples or pooled samples ([P450 SCC; DF, n=42; SF, n=38][P450 3β-HSD; DF, n=41; SF, n=38]). Briefly, 5 μg of total RNA was diluted in 250 μl of 50% formamide-6%formaldehyde-20 nM Tris, pH 7.0, and heated at 65°C for 5 minutes. To each sample was added 250 μl of 20 X SSC (1 X SSC=150 mM NaCl and 15 mM sodium citrate, pH 7.0), followed by vortex mixing and brief centrifugation in a microcentrifuge. A Bio Trans nylon membrane presoaked for 5 minutes in distilled water and then for 10 minutes in 10 X SSC was positioned in a Minifold® microsample filtration device (Schleicher and Schuell, Inc. Keene, NH) following manufacturer's instructions. Each well received a filtration sequence of 500 μl 10 X SSC, the RNA sample and 500 μl 20 X SSC. Nylon filters were then air dried, baked at 80°C for 2 h and stored in heat sealable pouches (Kapak®, St. Louis Park, MN) at 4°C until hybridized.

Prehybridizations (30 min) and hybridizations (16 hrs) were performed in 0.5 M Na_2HPO_4-7% SDS-1% bovine serum albumin-10 mM EDTA (Church and Gilbert, 1984; Appendix 4) at 65°C (P450 SCC and P450 3β-HSD mRNA) or 60°C (28S rRNA).

Washing was performed at 65°C (P450 SCC and P450 3β-HSD mRNA) or 60°C (28S rRNA) for two periods of 15 min in 40 mM Na_2HPO_4-0.1% SDS.

cDNA Probes

The bovine P450 SCC cDNA containing the complete coding sequence was a gift from Dr. M. Waterman (Univ. of Texas, Dallas; John *et al.*, 1984), whereas the 1169 base pair (bp) bovine P450 3β-HSD cDNA was kindly provided by Dr. F. Labrie (Laval Univ., Quebec; Zhao *et al.*, 1989). Both cDNA probes have been shown to hybridize to mRNA obtained from granulosa and theca cells of bovine preovulatory follicles before the LH surge (Voss and Fortune, 1993a; Hinshelwood *et al.*, 1993; Xu *et al.,* 1994). To correct for potential unequal loading of the RNA and variations in transfer efficiency, hybridization to 28S rRNA (human [5028 bp DNA fragment], Gonzalez *et al.*,1985; kindly provided by Dr. R. Schmickel [Univ. Pennsylvania, Philadelphia]) was determined. The cDNAs were labeled with [α-^{32}P]deoxy-CTP (ICN Radiochemicals, Irvine, CA) by use of Multiprime DNA Labeling System (Amersham Int., UK; Appendix 5). The labeled cDNA probes were separated from unincorporated label using a Sephadex® G-50 gel filtration column (Pharmacia Biotechnology Inc., Piscataway, NJ).

Phosphor Screen Autoradiography

Phosphor screen autoradiography of nylon filters (Northern and Dot blot; 24-48 hr), scanning, digitalization and processing of the image were performed with a PhosphorImager Series 400 (Molecular Dynamics, Sunnyvale, CA). A Molecular Dynamics Software package (ImageQuant™ V3.3, 1993) was used to further process the data to obtain an image of the original radioactive sample in pixel values proportional to the amount of radioactivity. X-Ray films (X-OMAT-LS, Eastman Kodak, Rochester, NY) were exposed to nylon filters (Northern and Dot Blots) for 2 hours to 4 days. Photos of X-Ray autoradiography were utilized to document the presence and the size of single bands in Northern blots and the different intensity of hybridization in Dot blots.

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models Procedure (SAS®, 1988). The size of the DF and SF, the difference between the size of the DF and SF (dominance) and plasma P_4 were analyzed as a split plot design with repeated measures of each cow over time. The mathematical model used for these analyses included the main effects of experimental group (D5, D8, D12-I, D12+I), experimental period (INT or period 1 and OVX or period 2), day of estrous cycle, and the interactions of experimental group by period, experimental group by day of estrous cycle, period by day of estrous cycle, and experimental group by period by day of cycle. In addition, tests of homogeneity of regression for day trends among experimental groups and periods (Wilcox *et al.*, 1990) were performed on size of the DF, SF, dominance and number of follicles per class. The FF P_4 concentrations and densitometry readings of RNA blots probed for 28S rRNA were analyzed as a split plot design. The mathematical model used for these analyses included the main effects of experimental group, follicle and experimental period, and the interactions of experimental group by follicle, experimental group by period, follicle by period and experimental group by follicle by period. The content of P450 SCC and P450 3β-HSD mRNAs were analyzed with the same model, but using 28S rRNA content as a covariate. This adjusts each sample for unequal loading of

RNA or for variations in transfer efficiency. The *F* test for the main effect of experimental group, follicle and the experimental group by treatment interaction were performed using the means square of cow within experimental group and follicle as the error term. Orthogonal contrasts were used to test differences between experimental groups, follicles, and experimental groups by follicle interactions (D5&D8 vs D12-I&D12+I, D5 vs D8, D12-I vs D12+I, DF vs SF; Petersen, 1985). However, because of the structure of the data (missing observations within treatment-follicle categories), many of the tests of significance for orthogonal contrasts were not obtained when the complete model was used. Under these circumstances, a reduced model, where the main effect of follicle was not included, was used to obtain this information. When this reduced model was used, the *F* test for the main effect of experimental group was done using the means square of cow within experimental group as the error term. Dependent variables among experimental groups were tested for homogeneity of variances by Bartlett's test (Ostle, 1963) and for normality by the Kolomogorov-Smirnov tests (Ostle, 1963; SAS®, 1988). When the data were determined to fail these tests, an appropriate transformation was made (e.g. log 10). Responses are presented as LSM ± SEM for the experimental groups by follicle.

Results

Ovarian Follicular Dynamics

The pattern of growth of the DF during the first 12 days of the cycle was different for cows that had both ovaries (period 1) compared to those that had a single ovary (period

2). An experimental group by day interaction was detected ($P < .01$) for the size of the DF (Fig 3-2, top-left). The size of the DF had different patterns of growth in intact and in unilateral ovariectomized cows ($P < .01$; Fig. 3-2, top-left). The DF in unilateral ovariectomized cows emerged 2 d earlier (DE=2) compared to the DF of intact cows (DE=4). However growth rate of the DF was greater in intact cows because although the follicle emerged 2 day later, it achieved the same size in both groups by day 5. Moreover, SF size was smaller during the first 5 days of the estrous cycle in unilateral ovariectomized cows compared to intact cows ($P < .01$; Fig.3-2, top-right). An experimental group by day interaction also was detected for the difference in size between the dominant and the first subordinate follicle (i.e. dominance; $P < .01$; Fig 3-2, bottom). Dominance started approximately two days earlier (period 1, DE=4, period 2, DE=2) and was much stronger (up to d 5) in DF of unilateral ovariectomized cows compared to intact cows (Fig. 3-2, bottom; $P < .01$). Conversely, after d 5, size of the SF was bigger and size of the DF and dominance were smaller in unilateral ovariectomized cows compared to intact cows (Fig. 3-2, top-left, top-right and bottom). Although a lower number of class 1 follicles per cow was present in the second period ($P < .01$; Fig.3-3), this only represented the effect of having one vs two ovaries. No period or unilateral ovariectomy effect was detected when the average number of class 1 follicles per ovary was compared between periods 1 and 2. No changes were observed in the number of class 2 ($P < .27$) and class 3 follicles ($P < .58$) expressed as per cow between intact and unilateral ovariectomized cows.

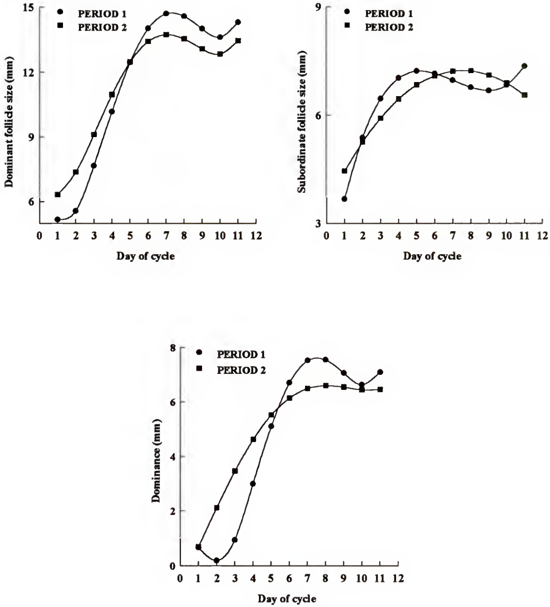

Figure 3-2. Pattern of growth of first wave DF (top-left), SF (top-right) and of dominance (difference in size between dominant and first subordinate follicle, bottom, center) during day 0 to day 12 of synchronized estrous cycles of non-lactating dairy cows.

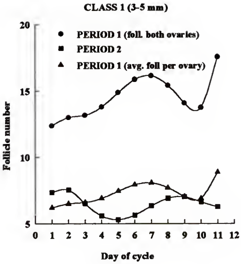

Figure 3-3. Number of class 1 (3-5 mm) follicles in period 1 (INT; both ovaries), period 1 (INT; average follicles per ovary) and period 2 (OVX) in non-lactating dairy cows.

For the overall experiment (excluding D12+I cows), the average size of the DF and SF are presented in Fig.3-4 (left) and the average number of class 1, class 2 and class 3 follicles are presented in Fig. 3-4 (right). The pattern of growth of the DF and SF are very similar up to d2.5 of the estrous cycle when selection and the emergence of the DF take place. Thereafter, whereas the SF remains stable in size, the DF continues to growth until d7 of the cycle, were it reaches a plateau, and then starts to decrease in size. The number of class 1 follicles increased early in the estrous cycle (d1-d3) and then decreased (d4-d6) whereas average number of class 2 follicles increased during the recruitment phase (d3-d4). On approximately d2.5-3.5, one class 3 follicle is selected which thereafter becomes the dominant follicle.

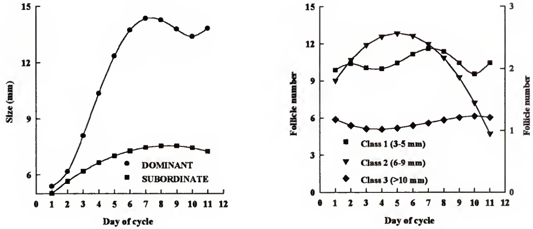

Figure 3-4. Average diameter of the DF and SF (left) and average number of class 1 (3-5 mm), class 2 (6-9 mm) and class 3 (>10 mm; right) in D5, D8 and D12-I cows.

Follicle Diameter at Ovariectomy

A period by follicle interaction was detected for follicle size at ovariectomy ($P<.03$). The diameter of the DF was not different for intact and for unilaterally ovariectomized cows (16.68±0.31 vs 16.66 ±0.30 mm; Fig 3-5, left), but SF of intact cows were smaller than those from unilaterally ovariectomized cows (6.69 ±0.37 vs 8.64±0.49 mm; Fig 3-5, left). When only DF were included in the statistical analysis, D5 DF were smaller than D8 DF (14.20±0.92 vs 17.29±0.97 mm, $P<.04$) and D12-I DF were smaller than D12+I DF (15.40±0.92 vs 19.80±0.92 mm, $P<.04$).

Conversely, SF at D5 and D12-I were larger than their cohorts at D8 (8.53±0.34 vs 7.30±0.39 mm, $P<.01$), and D12+I (7.93±0.47 vs 6.90±0.56 mm, $P<.11$).

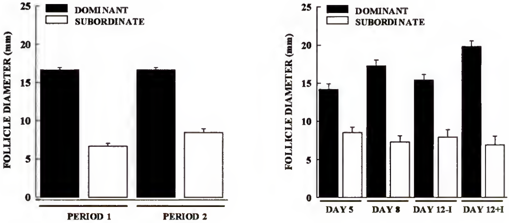

Figure 3-5. Diameter of DF and SF at ovariectomy in non-lactating dairy cows. Data represent least squares means (±SEM) of follicle diameter in two periods (1=INT, 2=OVX; left), or in four experimental groups (D5, D8, D12-I and D12+I; right).

Progesterone Concentrations in Plasma

Plasma P_4 concentrations were similar for intact and unilaterally ovariectomized cows ($P<.47$). However, an experimental group by day of cycle interaction was detected ($P<.01$; Fig 3-6, left). In D12+I cows, plasma P_4 declined from 8.04±0.39 ng/ml on d8 to 2.96±0.41 ng/ml on d12 whereas in D12-I cows, plasma P_4 remained at about 8.66 ±0.39 ng/ml. After d8, two types of plasma P_4 profiles were observed for the D12+I cows ($PGF_{2\alpha}$ injection and implant insertion) based upon whether CL regression was completed (e.g. $P_4 < 1$ng/ml) or not completed ($P_4 > 1$ ng/ml; n=11; $P < .05$; Fig 3-6, right). Cows of D12+I that underwent complete CL regression has plasma P_4 concentrations of 0.83±0.70 ng/ml (n=7) whereas those having incomplete CL regression had plasma P_4 concentrations of 3.03±0.56 ng/ml (n=7).

Progesterone Concentrations in Follicular Fluid

Concentrations of FF P_4 were higher for SF of intact cows than unilaterally ovariectomized cows (298.46±33.93 vs 65.45±23.92 ng/ml; Fig 3-7, left), but were not different in DF of either group (72.88±17.04 vs 76.08±17.04; period by follicle interaction, $P < .01$). Concentrations of P_4 in FF of DF and SF exhibited distinct changes among experimental groups (experimental group by follicle interaction, $P < .03$; Fig. 3-7, right). A separate analysis that included only DF indicated that D5 and D8 follicles did not differ in FF P_4 (34.41±38.27 vs 37.72±44.19 ng/ml, $P < .96$), but FF P_4 in that D12-I and D12+I differed (164.97 vs 60.88±41.92 ng/ml, $P < .09$). The same approach was utilized for the analysis of SF. Concentrations of P_4 in FF of D12+I SF were increased compared to those of D12-I follicles (311.93 vs 27.96±97.17 ng/ml, $P < .08$).

Ovarian follicles were classified as either atretic ($P_4 \geq 100$ ng/ml; ultrasonographic data (size, day of wave, dominant or subordinate) or non-atretic EA ($P_4 < 100$ ng/ml; ultrasonographic data (size, day of wave, dominant or subordinate). Table 3-1 depicts the frequency of follicles (DF vs SF) within each experimental group (D5, D8,D12-I and

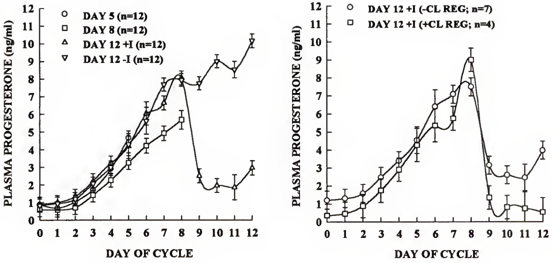

Figure 3-6. Plasma concentrations of P_4 during day 0 to day 12 of synchronized estrous cycles in dairy cows.

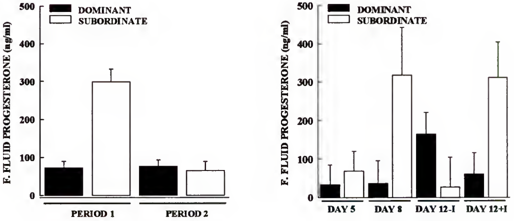

Figure 3-7. Follicular fluid concentrations of P_4 in first wave DF and SF of dairy cows. Data represent least squares means (±SEM) of follicular fluid steroid concentrations in two periods (1=INT, 2=OVX; left), or in four experimental groups (D5, D8, D12-I and D12+I; right).

Table 3-1. Frequency of follicles (dominant and subordinates) with P_4 concentrations < 100 ng/ml or ≥ 100 ng/ml in the four experimental groups studied (day 5, E_2 active; D8 transitional E_2 active; D12-I atretic and D12+I E_2 active-sustained).

	Day 5[a]		Day 8[b]		Day 12-I[c]		Day 12+I[d]	
P_4 (ng/ml)	<100	≥100	<100	≥100	<100	≥100	<100	≥100
DF[e]	12	0	9	1	6	5	9	2
SF[f]	11	1	6	4	11	0	9	2

[a]Day 5 E_2 active experimental group,
[b]Day 8 transitional E_2 active experimental group,
[c]Day 12-I atretic experimental group,
[d]Day 12+I E_2 active-sustained experimental group,
[e]Dominant follicle,
[f]Subordinate follicle,
P_4 concentrations (ng/ml) orthogonal contrasts:

Experimental group by follicle interaction ($P < .07$);

DF, Experimental group ($P < .16$), D5&D8 vs D12-I&D12+I ($P < .07$), D5 vs D8 ($P < .64$), D12-I vs D12+I ($P < .21$);

SF, Experimental group ($P < .25$), D5&D8 vs D12-I&D12+I ($P < .27$), D5 vs D8 ($P < .12$), D12-I vs D12+I ($P < .30$).

D12+I) with FF P_4 < or ≥ 100 ng/ml (experimental group by period interaction, $P<.07$). Day 5 and D8 DF, had higher frequency of follicles with P_4 <100 ng/ml compared to D12-I and D12+I DF (2/22 vs 15/22, $P<.07$). Furthermore, frequencies of DF with P_4 < 100 ng/ml were similar between D5 and D8 DF (12/12 vs 9/10, $P<.64$), and between D12+I and D12-I DF (9/11 vs 6/11, $P<.21$). The frequency of SF with P_4 ≥ 100 ng/ml were similar among experimental groups ($P<.25$). Therefore, utilizing ultrasonographic data (i.e. size, day of wave, dominant or subordinate) and P_4 concentrations in FF, the majority of D5, D8 and D12+I DF were classified as non-E_2 active (30/33) and less than half of D12-I DF were classified as atretic (5/11). Furthermore, the majority of D5, D8, D12+I and D12+I SF were classified as non-atretic E_2 active (37/44).

P450 SCC mRNA Content in Follicle Walls

Photographs of a Northern blot containing RNA from eight representative DF and of an ethidium bromide stained agarose gel are depicted in the top and bottom of Fig. 3-8. A single P450 SCC hybridization signal was observed at 2.0 Kb. The pattern of ethidium bromide staining of the 18S and 28S bands was of equal intensity across the lanes.

Preliminary analysis indicated that relative 28S rRNA contents did not differ for DF and SF (333.53 ± 24.99 vs 295.81 ± 27.47 AU, $P<.32$). Therefore, P450 SCC mRNA content (AU/5μg total RNA) in each follicle wall was adjusted for the 28S rRNA content (AU/5μg total RNA) of the same sample. Steady state levels of P450 SCC mRNA determined by RNA dot blot analysis are shown in Fig. 3-9.

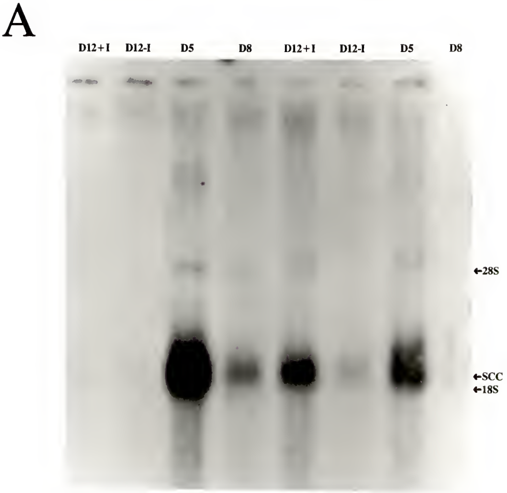

Figure 3-8. Northern blot analysis of P450 Side chain cleavage. Ten μg of total RNA extracted from first wave DF at D5, D8, D12-I and D12+I were loaded per lane. A. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 SCC cDNA. The hybridization signal was present at 2Kb. B. Picture of ethidium bromide stained gel demonstrating equal loading of RNA in each lane.

B

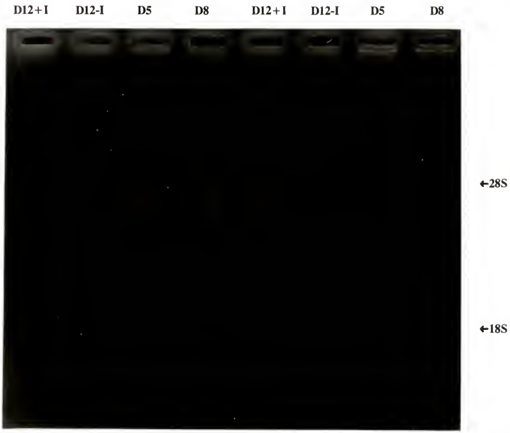

Figure 3-8. continued

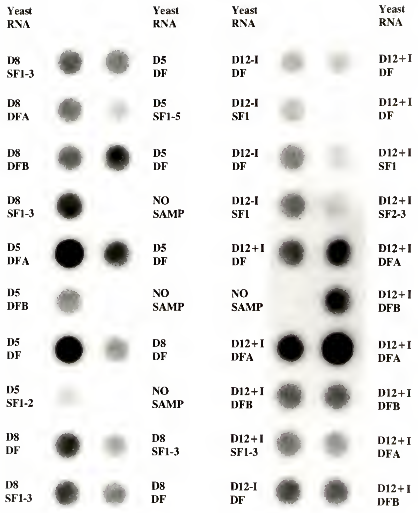

Figure 3-9. Dot blot analysis of P450 Side chain cleavage. Five μg of total RNA extracted from first wave DF or SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 SCC cDNA.

Steady state levels of P450 SCC mRNA were increased in unilaterally ovariectomized compared to intact cows (27.10±2.96 vs 17.85±2.80 AU, $P<.04$; Fig. 3-10, left). Although, DF had approximately double the content of P450 SCC mRNA compared to SF (28.08±2.93 vs 16.87±4.26 AU, $P<.02$; Fig. 3-10, right) this difference resided mainly in the D5 DF.When only DF were included in the analysis, a main effect of experimental treatment was detected ($P<.04$). D5 and D8 DF had a higher expression level of P450 SCC mRNA compared to D12-I and D12+I DF (35.64±7.27 vs 20.51±7.25 AU, $P<.06$). The P450 SCC mRNA content at D5 was greater than at D8 DF (46.38±6.43 vs 24.91±8.12 AU, $P<.05$), but no difference was detected between D12-I and D12+I DF (18.90±8.17 vs 22.12±6.33, $P<.76$). The P450

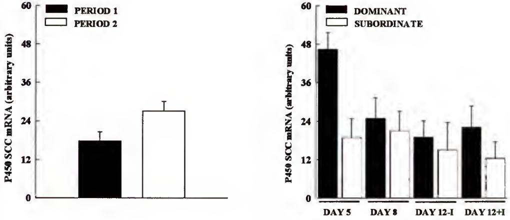

Figure 3-10 Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 SCC cDNA to mRNA from first wave DF and SF of non-lactating dairy cows.

SCC mRNA levels decreased in a linear fashion in the process of atresia of the dominant follicle (P450 SCC mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF > D12-I atretic DF; $Y=61.63+[-3.78X]$; $R^2=0.84$). However, when D12+I DF (E_2 active sustained) rather than D12-I DF (atretic) were included in the regression analysis, a reduction in the slope of the equation was observed (P450 SCC mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF ≤ D12-I atretic DF; $Y=58.74+[-3.31X]$; $R^2=0.76$). There was no effect of experimental treatment on the steady state levels of P450 SCC mRNA in SF ($P<.49$).

P450 3-βHSD mRNA Content in Follicle Walls

Photographs of a Northern blot of RNA from eight representative DF probed for P450 3β-HSD mRNA and an ethidium bromide stained agarose gel are shown in the top and bottom, respectively, of Fig. 3-11. A single P450 3-βHSD hybridization signal was observed at a migration position of 1.7 Kb.

Expression levels of 28S rRNA did not differ for DF and SF (313.10±12.99 vs 284.46±13.98 AU, $P<.14$). Therefore, P450 3-βHSD mRNA content in each follicle wall was adjusted for the 28S rRNA content of the same sample. Steady state levels of P450 3-βHSD mRNA as determined by RNA dot blot analysis are shown in Fig 3-12.

The overall expression of P450 3-βHSD mRNA was increased in unilaterally ovariectomized cows compared to intact cows (305.05±27.56 vs 239.70±25.73 AU, $P<.10$; Fig. 3-12, left). Abundance of P450 3β-HSD mRNA did not follow the same pattern for DF and SF among groups (experimental group by follicle interaction, $P<.02$; Fig 3-12, right). Analysis of the DF P450 3β-HSD mRNA levels using the reduced

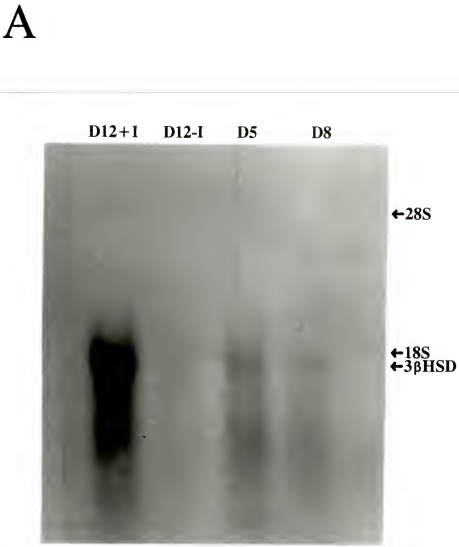

Figure 3-11. Northern blot analysis of P450 3β-hydroxysteroid dehydrogenase. Ten μg of total RNA extracted from first wave DF at D5, D8, D12-I and D12+I were loaded per lane. A. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 3β-hydroxysteroid dehydrogenase cDNA fragment (1169 bp). The hybridization signal was present at 1.7 Kb. B. Picture of ethidium bromide stained gel demostrating equal loading of RNA in each lane.

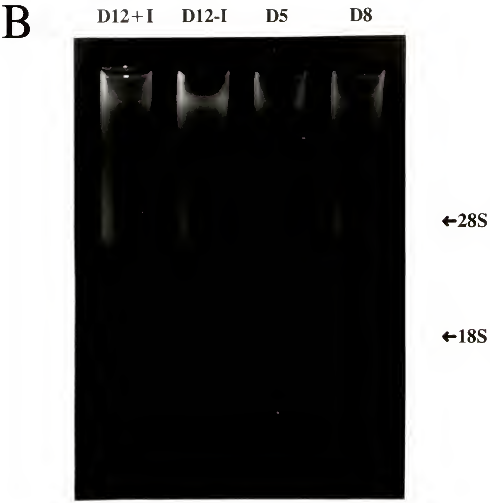

Figure 3-11. continued

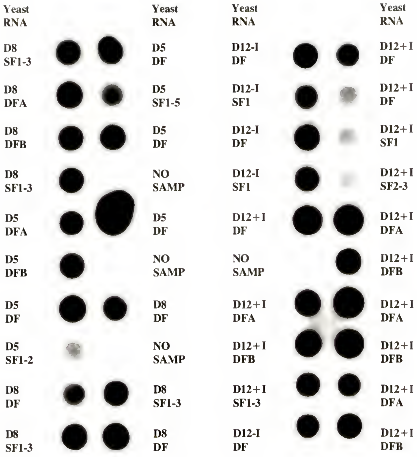

Figure 3-12. Dot blot analysis of P450 3β-hydroxysteroid dehydrogenase. Five μg of total RNA extracted from first wave DF or SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 3β-HSD cDNA.

mathematical model indicated experimental group differences ($P<.04$). Day 5 DF had higher expression of P450 3β-HSD mRNA compared to DF at D8 (416.28±48.99 vs 237.47±57.55 AU, $P<.03$). Likewise, P450 3β-HSD mRNA was greater for D12+I compared to D12-I DF (393.68±51.81 vs 194.90±66.11 AU, $P<.04$). The P450 3β-HSD mRNA levels decreased in a linear fashion in the process of atresia of the dominant follicle (P450 3β-HSD mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF > D12-I atretic DF; $Y=506.79+[-26.93X]$; $R^2=0.76$). However, when D12+I DF (E_2 active sustained) rather than D12-I DF (atretic) were included in the regression analysis, a quadratic equation described the changes in P450 3β-HSD (P450 3β-HSD mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF ≤ D12-I atretic DF; $Y=1261.18+[-240.91X]+[14.00X^2]$; $R^2=0.98$). Experimental treatment did not affect the levels of P450 3β-HSD mRNA in SF ($P<.25$).

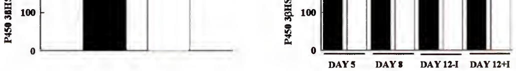

Figure 3-13. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 3β-HSD cDNA to mRNA from first wave DF and SF of non-lactating dairy cows.

Discussion

In this study, an experimental model is described to obtain first wave DF and SF at defined stages of development (D5=E_2 active DF, D8=transitional E_2 active DF, D12-I=atretic DF and D12+I=E_2 active sustained DF). This model eliminates a great deal of variation observed in follicles obtained from slaughterhouse ovaries where follicles are at different and unknown stages of development and allows a more detailed study of the various endocrine and molecular aspects that regulate dominance and atresia in individual follicles. This study documents changes in steady state levels of P450 SCC and P450 3β-HSD mRNA content and follicular fluid P_4 concentrations occurring in first wave dominant and subordinate follicles during four very distinct stages of follicular development. Levels of mRNA for P450 SCC and P450 3β-HSD did not parallel the increased concentrations of FF P_4 in atretic D12-I DF.

In agreement with previous studies (Ginther *et al.*, 1989; Savio *et al.*, 1990; Badinga *et al.*, 1992), the size of the DF increased from d 1 to d 8 and did not change in size thereafter until d 12 (Fig. 3-2, top-left). However, in contrast with previous studies (Badinga *et al.*, 1992), the DF of unilaterally ovariectomized cows had a greater diameter during the first 5 d of the cycle and dominance was initiated 2 days earlier (Fig. 3-2, top left and bottom center). This observation is further supported by a decrease in the number of class 1 follicles during the same period of time (Fig. 3-3) and by no difference in the number of class 2 follicles (e.g. follicles are not able to enter the upper classes). Conversely, after d 5 of the estrous cycle, the DF of unilaterally ovariectomized cows was smaller and less dominant compared to intact cows. This observation is supported by a bigger SF in these cows. Collectively, these data indicate a weaker ovarian negative feed-back as a result of removing the ovary bearing the DF in the second period; this alteration induced the DF to emerge earlier and grow faster during the earlier period of dominance. However, this initial difference disappeared after d 5, and may be due to the DF itself producing a stronger negative feed-back via inhibin secretion which very likely slowed down its own rate of growth. In intact cows, the exact mechanisms whereby the DF suppresses growth of SF are not yet well established. However FSH (Findlay *et al.*, 1992), LH (Savio *et al.*, 1993), inhibins (Findlay *et al.*, 1992; Findlay, 1993), E_2 (Findlay *et al.*, 1992), insulin-like growth factor I (IGF-I; Hammond *et al.*,1991; Giudice, 1992) and the IGFs binding proteins (IGFBPs; Hammond *et al.*,1991; Giudice, 1992) have been suggested to be responsible for regulation of ovarian follicular growth. It is very likely that these hormones and growth factors also are involved in the expression of enhanced dominance observed in unilaterally ovariectomized cows. Dominance appeared to be greater for the first 5 days of the cycle in cows bearing a single ovary (Fig. 3-2, bottom). Associated with these size differences between DF and SF attributed to dominance between periods was a reduction in the number of class 1 follicles (Fig. 3-3). Follicular dynamics in intact cows clearly shows that expression of dominance is associated with a decrease in of class 2 follicles (Lucy *et al.*, 1992). Follicular dynamics of cows bearing only one ovary may be altered such that with greater dominance follicular growth is altered in the class of smaller follicle size (class 1). The size of the DF and SF, and the number of class 1, 2 and 3 follicles in D5, D8 and D12-I experimental groups are presented in Fig. 3-4 (left and right) and agree with those reported earlier by Ginther *et al.* (1989), Savio *et al.* (1990), Badinga *et al.* (1992) and Lucy *et al.* (1992).

The DF had the same diameter at the time of ovariectomy in unilaterally ovariectomized and intact cows, but SF were larger in unilaterally ovariectomized cows (Fig. 3-5). This increase in diameter at time of ovariectomy of the SF in the second period was due to the presence of a less active DF during the last 7 days of the cycle (Fig.3-2, top-left). This also is supported by the lower FF P_4 concentrations observed during the second period in SF which is indicative of a lower degree of atresia (Fig. 3-7, left). Collectively these data indicate that the DF in the second period grew faster up to d 5 and expressed a stronger dominance when compared to the DF of the first period. However, this effect was reversed during the plateau period of the wave, and by the time of ovariectomy the D12-I and D12+I SF in the second period had a lower level of atresia as measured by a decrease in P_4 concentrations compared to period 1. Badinga *et al.* (1992) did not detect differences in follicle diameter or in FF P_4 of DF and SF of intact or unilaterally ovariectomized cows, and measurement of dominance was not reported.

Plasma P_4 profiles agree with those reported elsewhere (Sirois and Fortune, 1988; Fortune *et al.,* 1991; Badinga *et al.,* 1992; de la Sota *et al.,*1993) during the first 5, 8 or 12 days of the estrous cycle for cows included in the D5, D8 and D12-I experimental groups (Fig. 3-6, left). Complete regression of the CL was not achieved in some cows of the D12+I group (Fig. 3-6, right).

Concentrations of P_4 and E_2 control the development of functional oxytocin receptors during a normal cycle, and it appears that normal response of the endometrium to oxytocin requires prior exposure to progesterone (Vallet *et al.*, 1990; Beard and Hunter, 1994). Premature luteal regression can result from an early activation of the normal luteolytic mechanisms as a result of high concentrations of uterine oxytocin receptors during the early luteal phase (Hunter *et al.*, 1990). This early activation may take place either due to low P_4 priming (no downregulation of E_2 receptors) or to higher concentrations of E_2 from the DF. Higher concentrations of E_2 from active DF (d3-4) could have induced higher oxytocin receptor numbers allowing complete CL regression in response to the luteolytic dose of $PGF_{2\alpha}$ used (see Chapter 4). Plasma P_4 concentrations during the first 7 days of the estrous cycle did not differ between cows experiencing complete or incomplete CL regression following injection of $PGF_{2\alpha}$ on d8 (Fig. 3-6, right). Another factor leading to incomplete CL regression in seven cows of the D12+I group could have been an insufficient dose of $PGF_{2\alpha}$ at the time of implant insertion. This scenario is unlikely since the same dose of $PGF_{2\alpha}$ was use for the synchronization of all animals and luteolysis was induced in all cows (n=24, two synchronization periods) as confirmed by ultrasonography and detection of estrus.

Dominant follicles obtained at D5, D8 and D12+I had FF P_4 concentrations below 100 ng/ml and were classified as non-atretic E_2 active (Ireland and Roche, 1983; Grimes *et al.*, 1987; Martin *et al.*, 1991) whereas half of D12-I DF had FF P_4 concentrations above 100 ng/ml and hence were classified as atretic or E_2 inactive (Ireland and Roche, 1983; Grimes *et al.*, 1987; Martin *et al.*, 1991; Fig. 3-7, right). Surprisingly, the majority of D5, D8 and D12+I SF had FF P_4 concentrations below 100 ng/ml and were classified as non-atretic E_2 active (Ireland and Roche, 1983; Grimes *et al.*, 1987; Martin *et al.,* 1991). However, these SF were clearly subordinate and atretic based upon the ultrasonographic data (size, day of wave, dominant or subordinate) and the absolute P_4:E_2 ratio (PERT; Ireland and Roche, 1983; Chapter 6; Fig. 6-1, right; Table 6-1). Therefore, classifying individual follicles on the basis of FF concentrations of P_4 alone and ultrasonographic data appears to yield too many follicles as E_2 active when actually the same follicles are classified as truly atretic or E_2 inactive follicles based upon the P_4:E_2 ratio. Conversely, the use of a threshold of 50 ng/ml of FF E_2 and ultrasonographic data (Chapter 4; Fig. 4-2, right; Table 4-1) yielded the same results as those obtained using the E_2:P_4 ratio (EPR; Grimes *et al.,* 1987; Martin *et al.,* 1991; Chapter 5; Fig. 5-1, right; Table 5-1), and demostrated that a single absolute value of FF estradiol value could be used to classify follicles as either E_2 active or E_2 inactive.

Follicular fluid concentrations P_4 in SF were two-fold higher than in DF (Fig. 3-7, right). The FF concentrations of P_4 in D8 DF were quite similar to those present in D5 DF; however, a four-fold increase FF P_4 concentrations was present in D12-I DF (Fig. 3-7, right). This agrees with previous studies (Ireland and Roche, 1983; McNatty *et al.,* 1984; Martin *et al.,*1991; Badinga *et al.,* 1992) and the marked increase in FF P_4 is indicative of atresia which did not occur in D12+I DF where FF P_4 concentrations remained at concentrations comparable to those observed in DF at D5. The induction of CL regression and insertion of a progestin implant clearly changed the pattern of follicle turnover in D12+I cows compared to D12-I cows. Although follicle development in cattle is primarily regulated by gonadotrophic hormones (Richards, 1980; Ireland and Roche, 1987, Lucy *et al.,*1992), the terminal stages of follicular development may be regulated at the ovarian level by the presence of an E_2 active dominant follicle (Savio *et al.*, 1993). High amplitude and low frequency LH pulses around d 8-9 of the estrous cycle are not sufficient to maintain continued growth and function of the DF and under normal circumstances this first wave DF becomes atretic. However, this process of atresia for the first wave DF was reversed in the D12+I cows by changing LH pulsatility to a pattern of high frequency with induction of luteolysis and the insertion of a progestin implant (Savio *et al.*, 1993). Maintenance of dominance in D12+I DF also is supported by the low FF P_4 concentrations of the D12 DF and that SF in D12+I cows had a 10 fold increase in FF P_4 compared to the low FF P_4 concentrations of D12-I SF (Fig. 3-7, right). Conversely, the low level of atresia as reflected by low FF P_4 concentrations observed in D12-I SF is explained by turnover of the DF and recruitment of a new wave of follicles when concentrations of plasma P_4 were low.

Northern blot analysis of individual dominant follicles detected a single hybridization signal at 2.0 Kb for P450 SCC (Fig. 3-8) and at 1.7 Kb for P450 3β-HSD (Fig. 3-11). Similar sizes have been reported for P450 SCC (Rodgers *et al.*,1987; Voss and Fortune, 1993a) and P450 3β-HSD (Voss and Fortune, 1993a; Zhao *et al.*, 1989) mRNAs in thecal and granulosa cells from bovine follicles. Rodgers *et al.* (1987) compared levels of P450 SCC in bovine follicles and corpora lutea obtained at the abattoir. Levels of P450 SCC in follicles were similar to those for corpora lutea in the early luteal phase, but were 12 fold lower than for mid luteal phase corpora lutea. Similar results in

P450 SCC and P450 3β-HSD mRNA levels were found by Hinshelwood *et al.* (1993) for bovine follicles and corpora lutea obtained from abattoir.

A major objective of this chapter was to determine if the pattern of change in P_4 concentrations in DF and SF associated with atresia of the dominant follicle were also associated with parallel changes in gene expression of P450 SCC and P450 3β-HSD enzymes. Steady state levels of P450 SCC mRNA of D8, D12-I and D12+I DF, were one-half of those present in D5 DF. The mRNA levels of P450 3β-HSD followed this same pattern with the exception of D12+I DF in which mRNA levels were comparable to levels present in D5 DF. The mRNA levels of SF for both steroidogenic enzymes remained unchanged across the four experimental groups. In previous studies, levels of mRNA for P450 SCC and P450 3β-HSD mRNA in bovine follicles have been reported for ovaries obtained from the abattoir without dating precisely the stage of follicle development (Funkenstain *et al.,* 1983, 1984; Rodgers *et al.,* 1986ab, 1987; Hinshelwood *et al.,* 1993). More recently, follicles obtained from ovaries at precise stages of the estrous cycle have been utilized for mRNA studies. Voss and Fortune (1993a) studied mRNA levels of P450 SCC and P450 3β-HSD in preovulatory DF around the LH surge whereas Xu *et al.* (1994) focused studies on follicles obtained at different stages of development during the first 10 days of the first follicular wave. However, no atretic DF nor SF were included in the first study, and neither steroidogenic status nor SF were examined in the second study. The present results do not support the hypothesis that the increase in FF P_4 production observed during the process of atresia of the DF (Fig. 3-7, right) was paralleled with an increase in the levels of P450 SCC and P450 3β-HSD mRNA in D12-I DF (Fig. 3-10, right; Fig. 3-13). The P450 SCC and P450 3β-HSD mRNA levels in D12-I DF remained unchanged compared to D8 DF whereas FF P_4 concentrations increased four-fold. However, P450 SCC and P450 3β-HSD levels were markedly higher in D5 DF associated with early dominance in E_2-active DF. Conversely, in D12+I DF, where dominance is maintained, P450 3β-HSD mRNA levels were increased. This last observation could be interpreted as a necessary increase in mRNA levels to convert dehydroepiandrosterone to androstenedione to produce substrate for E_2 production by granulosa cells of the DF. Collectively these results indicate that the increase in P_4 observed during atresia of D12-I DF could be due to a loss of the capability of these follicles to further metabolize pregnenolone to androstenedione and 17 β-estradiol through the action of P450 17α-HYD and P450 ARO. When this takes place, pregnenolone is no longer utilized for androstenedione production, and thus can be converted to progesterone by P450 3β-HSD. However, this increase in P_4 production or metabolism of pregnenolone to P_4 is not reflected by an increase in P450 3β-HSD gene expression. In D12+I DF, where a high LH pulse frequency is maintained, the capability of follicles to further metabolize pregnenolone to androstenedione and 17 β-estradiol through P450 17α-HYD and P450 ARO is maintained and P_4 concentrations in FF remained low (Fig. 3-7, right). Moreover, inhibition of the conversion of pregnenolone to progesterone by high concentrations of FF E_2 would suppress P_4 production and provide additional pregnenolone to be metabolized to androgens (Fortune and Hansel, 1979). Voss and Fortune (1993a) proposed a similar mechanism to account for an increase in FF P_4 concentrations without a concomitant increase in mRNA levels for P450 SCC and for P450 3β-HSD in periovulatory follicles.

The same temporal pattern was observed between FF P_4 concentrations and expression of P450 SCC in SF. For example, at D8 and D12+I there was a ten-fold increase in FF P_4 with no concomitant increase in mRNA levels of either steroidogenic enzymes. In SF at D8 and D12+I, a lower number of LH receptors (Hanukoglu, 1992) and presence of growth factors such as (fibroblast growth factor, transforming growth factor-β and epidermal growth factor) inhibitory to P450 17α-HYD activity (Simpson *et al.,* 1992) may be responsible for the suppression of P450 17α-HYD activity and further utilization of pregnenolone for P_4 production.

By sustaining development of the first wave DF (D12+I), atresia was blocked as indicated by maintenance of a low P_4 intrafollicular environment (Fig. 3-6). This decrease in P_4 was not associated with any change in P450 SCC levels but was inversely associated with an increase in P450 3β-HSD. However, this increase in P450 3β-HSD is not likely associated with enhanced FF P_4 since such a relationship was not evident in DF of D12+I or in D5 DF in which high P450 3β-HSD was associated with low FF P_4 (Fig. 3-6). This alteration in P450 3β-HSD is probably associated with enhanced E_2 secretion by the DF to be discussed in Chapter 4. As discussed earlier, P450 SCC and P450 3β-HSD are expressed in both theca and granulosa cells (Voss and Fortune, 1993a). Our present results are on follicular walls and a different pattern of expression of P450 SCC and P450 3β-HSD was detected in D12+I DF. Luteinization and large increases in P_4 are thought to be associated with changes in granulosa cells. The present results would suggest that an increase in FF P_4 in D12-I DF is not associated with the typical luteinization response induced by LH (Voss and Fortune, 1993a) in that neither P450 SCC and P450 3β-HSD were increased in parallel with the increase in FF P_4. The subtle changes in steroid metabolism and/or enzymatic activity must be occurring independently of marked changes in gene expression of P450 SCC and P450 3β-HSD in D12-I DF. In contrast, a rescue of the DF by inducing a low P_4 environment in plasma after injection of $PGF_{2\alpha}$ (D12+I) resulted in stimulation of P450 3β-HSD. It is likely that this observation represents induction of P450 3β-HSD activity associated with a stimulation of E_2 secretion and that this occurs in theca cells based upon the A_4 and E_2 responses described in Chapter 4 and the fact that no P_4 increase was detected in FF (Fig. 3-6).

Sizes of the dominant follicle (Fig. 3-2, top-left) and dominance (difference in size between the dominant and the first subordinate follicle; Fig. 3-2, bottom) were greater during the first five days of the second experimental period (unilateral ovariectomized) compared to the first five days of period 1 (intact). Conversely, size of the subordinate follicle was smaller during the first five days of period 2 compared to period 1. Furthermore, steady state levels of P450 SCC and P450 3β-HSD mRNA were higher in unilaterally ovariectomized (period 2) cows compared to intact cows (period 1; Fig. 3-10, left; Fig. 3-13, left). Therefore, presence of a more dominant follicle during the first five days of the cycle in the second period was coupled with higher gene expression of P450 SCC and P450 3β-HSD during the same time. The P450 SCC enzyme is necessary for conversion of cholesterol to pregnenolone and P450 3β-HSD enzyme is necessary for conversion of DHEA to A_4 in theca and granulosa cells. This view is further supported by similar increases of P450 17α-HYD (Chapter 4; Fig. 4-6, left) and P450 ARO (chapter 4; Fig. 4-9, left) mRNA levels to support steroidogenesis in these follicles and by increases in α-bInhibin mRNA levels necessary to exert dominance by feed-back inhibition at the pituitary level and/or ovarian level (Chapter 5, Fig. 5-12, left). Although we were able to detect differences among experimental periods in gene expression of α-bInhibin, the four steroidogenic enzymes, dominant and subordinate follicle sizes and dominance, we were not able to detect similar differences in the product of these genes (i.e. FF concentrations of P_4, A_4, E_2 and α-bInhibin). It is very likely these lack of correlations between gene expression and steroid hormones in FF could be explained by postranslational mechanisms that affect mRNA translation or by mechanisms that affect enzyme activities such as cofactor availability or postranslational modification of the enzymes (Hanukoglu, 1992).

The following conclusions can be drawn from the present study: 1) an experimental model is described to obtain individual first wave dominant and subordinate follicles at precise stages of follicle development that eliminates potential variation observed in follicles obtained from the abattoir; this permitted a detailed study of the various endocrine and molecular aspects that regulate dominance and atresia of bovine ovarian follicles, 2) the majority of D5, D8 and D12+I DF were classified correctly as non-atretic E_2 active follicles whereas less than half of the D12-I and SF were classified as atretic, 3) although the follicular fluid concentration of P_4 increased with atresia of the first wave DF, steady state levels of P450 SCC and P450 3β-HSD decreased in a linear fashion with atresia of the first wave DF; hence, other mechanisms such as increased enzyme activity , cofactor availability and postranslational modifications of the enzymes may account for the increase in FF P_4 concentrations in the face of decrease in steady state levels of P450 SCC and P450 3β-HSD mRNA levels in these same follicles.

CHAPTER 4

ANDROSTENEDIONE AND 17β-ESTRADIOL CONCENTRATIONS AND mRNA LEVELS FOR P450 17α-HYDROXYLASE-17,20 LYASE AND P450 AROMATASE IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES

Introduction

The conversion of cholesterol to pregnenolone by P450 SCC in mitochondria is the first and rate-limiting step in the synthesis of steroid hormones in bovine preovulatory follicles (Hanukoglu, 1992; Miller, 1988), and is activated by LH (Fortune, 1986). Pregnenolone is further converted to 17-hydroxy-pregnenolone and DHEA by P450 17α-HYD through the Δ^5 pathway or to P_4 by P450 3β-HSD (Fortune, 1986; Fortune and Quirk, 1988; Hanukoglu, 1992). Dehydroepiandrosterone is metabolized further to A_4 by P450 3β-HSD in theca interna cells (Fortune, 1986; Fortune and Quirk, 1988; Hanukoglu, 1992). In granulosa cells, A_4 acts as the predominant substrate for aromatization by P450 ARO and is converted to E_2 supporting the concept of the two cell theory (Falk, 1959; Fortune, 1986; Fortune and Quirk, 1988; Hanukoglu, 1992; Lacroix et al., 1974). In the past, several studies attempted to classify first wave follicles as EA (dominant) or EI (atretic) based upon the EPR (EPR > 1, EA), or PERT (PERT > 1, EI or atretic) in FF (Ireland and Roche, 1983; Martin *et al.,* 1991; Ireland and Ireland, 1994), macroscopic appearance of the follicular surface (Grimes and Ireland, 1986), or the comparison of histological and non-histological indices of atresia (Grimes *et al.,* 1987). Furthermore, using absolute FF E_2 concentrations, Ireland and Roche (1983) classified bovine follicles as EA (non-atretic; $E_2=141\pm35$ ng/ml) or EI (atretic; $E_2=8\pm2$ ng/ml) whereas Ireland and Ireland (1994) classified bovine follicles as E_2 active ($E_2=99\pm32$ ng/ml), atretic ($E_2=19\pm15$ ng/ml), and highly atretic ($E_2=0.1\pm0.02$ ng/ml). Echternkamp *et al.* (1994) used a threshold of 50 ng/ml of E_2 in FF of porcine follicles to classify them as either EA (non-atretic) or EI (atretic). Collectively, these studies indicate that ovarian follicles could be classified as EA (non-atretic) or EI (atretic) based upon FF E_2 concentrations. Hence, ovarian follicles with FF E_2 concentrations above 50 ng/ml could be classified as EA whereas those with FF E_2 concentrations below 50 ng/ml could be classified as EI or atretic.

Daily ultrasonographic observations of bovine ovarian follicles characterized patterns of follicle development and permitted identification of the dominant and cohort of subordinate follicles in bovine ovaries as early as day 4 of the estrous cycle (de la Sota *et al.,* 1993; Ginther *et al.,* 1989; Lucy *et al.,* 1992; Savio *et al.,* 1988, 1990; Sirois and Fotune, 1988). The combination of ultrasonography and FF steroidal profiles has provided the opportunity to describe more accurately the pattern of growth and the endocrine and biochemical differences between first wave DF and SF at days 5, 8 and 12 of the estrous cycle (Badinga *et al.*, 1992). In the latter study, A_4 and E_2 concentrations of FF and aromatase activity in follicle walls were measured to characterize endocrine and biochemical characteristics of first wave DF and SF. Aromatase activities were comparable between D5 and D8 DF; however, FF E_2 concentrations declined markedly from D5 to D8. Both responses were further diminished by D12 in DF. This apparent discrepancy between aromatase activity and E_2 concentrations in FF of D8 DF was interpreted as a lack of androgen precursor to support estradiol synthesis, since when testosterone was added to the follicle walls during the incubation E_2 synthesis was sustained in D8 DF. Androgen synthesis by cells of the theca interna is highly dependent on LH pulse secretion (Hansel and Convey, 1983; McNatty *et al.*, 1984). Thus it is very likely that a reduction in androgen availability for aromatization may take place when LH pulse frequency declines during the transition from metestrus to diestrus during the estrous cycle (Rahe *et al.*, 1980). Moreover, loss of dominance of the first wave dominant follicle was associated with a decrease in the size of SF and decreases in FF E_2 with the concomitant increases in FF P_4 concentrations of DF (Badinga *et al.*,1992). If the low LH pulse frequency present at mid-cycle is increased by induction of CL regression and insertion of a progestin implant on d8 of the estrous cycle, the first wave DF continues to grow and maintains its dominance for a period of 15 d (Savio *et al.*, 1993). Collectivelly, these two experiments indicate that increased LH secretion could counter the process of atresia by maintaining thecal production of A_4 for synthesis of E_2 in granulosa cells of the dominant follicle.

Rodgers *et al.* (1986a,b, 1987) and Hinshelwood *et al.* (1993) reported levels of mRNA, protein, and activities of P450 17α-HYD and P450 ARO enzymes in bovine follicles and CL obtained from slaughterhouse ovaries. Voss and Fortune (1993b) have studied mRNA levels of P450 17α-HYD and P450 ARO in granulosa and theca cells of preovulatory follicles before and after the LH surge. Before the LH surge, expression of P450 17α-HYD mRNA was limited mostly to theca cells whereas expression of P450 ARO mRNA was limited to granulosa cells. However, after the LH surge both messages decreased by 96 and 94%, respectively in each cell type. Follicular fluid A_4 and E_2 concentrations followed a similar pattern to the changes in the steroidogenic enzymes. A different approach was taken by Xu *et al.* (1994) who characterized mRNA levels of P450 17α-HYD and P450 ARO of ovarian follicles by in situ hybridization during different stages of follicle development. P450 ARO was localized in granulosa cells of follicles ≥4 mm obtained at 0, 2, 4, 6, 8 and 10 d after initiation of the first wave of follicle development whereas P450 17α-HYD was localized in theca interna cells of all antral follicles. However, in this study no description of steroid concentrations in follicular fluid nor stage of follicle development (i.e. whether follicle was dominant or atretic) were available to integrate these with inferred changes in steroidogenic enzymatic gene expression.

A detailed study that compares mRNA levels of P450 17α-HYD and P450 ARO enzymes in DF and SF at defined stages of follicular development and during programmed changes in follicle turnover is lacking. Therefore, utilizing an experimental model described in Chapter 3, two main objectives were undertaken. The first objective was to study qualitative and quantitative changes in mRNA levels of P450 17α-HYD and P450 ARO in DF and SF at four defined stages of follicle development. In addition, it was tested whether expression of mRNAs encoding P450 17α-HYD and P450 ARO would decrease in a linear fashion during the transition from an E_2-active DF (D5) to transitional E_2 active DF (D8) and decrease further with atresia of the DF (D12-I). It also was hypothesized that expression of mRNAs for these steroidogenic enzymes would be maintained or increased under experimental conditions in which function of the DF is sustained by enhancing LH pulse frequency (D12+I DF). The last hypothesis to be tested was that the observed reduction in A_4 production in FF would be paralleled by a reduction in P450 17α-HYD gene expression in transitional E_2 active DF (D8).

The second objective of this chapter was to study biochemical and endocrine interrelationships among DF at different stages of dominance and atresia. Data presented in this chapter and in Chapter 3 were integrated into one analysis to quantify these relationships between variables studied by developing partial regression coefficients. These interrelationships were developed using simple correlations, partial correlations obtained from multivariate analysis of variance, and standardized regression coefficients obtained from stepwise regression analyses.

Material and Methods

The reader is referred to the material and methods section of Chapter 3 for a detailed description of the experimental animals and reproductive management, ultrasonographic examination and blood collection, tissue collection and handling, RNA isolation, Northern and dot blot analyses, phosphor screen autoradiography and statitistical analyses utilized in the present section.

Steroid Hormone Assay of Plasma and Follicular Fluid

Concentrations of FF A_4 were assayed in 100 μl aliquots (0.5 to 2.5 μl native FF equivalents; DF, n=44; SF, n=44) of unextracted FF by RIA (TKAS2, Diagnostic Product Co. Kit, Los Angeles, CA; Badinga *et al.*, 1992). The assay sensitivity was 0.02

ng/ml and the intraassay coefficient of variation (CV) was 8.3%. E_2 concentrations were assayed in 100 μl aliquots (0.5 to 2.5 μl native FF equivalents) of unextracted FF (Thatcher *et al.*, 1991) and in 1.0 ml aliquots of extracted plasma (Badinga *et al.*, 1992). Assay sensitivities were 2.0 pg/ml E_2 in FF and 1.25 pg/ml E_2 in plasma samples. The intra- and inter-assay coefficient of variation were 12.83% and 13.58% for assays of FF and 8.66% and 9.13% for assays of plasma, respectively. Ovarian follicles were further classified as either EA ($E_2 \geq 50$ ng/ml [Echternkamp *et al.*, 1994]; ultrasonographic data [size, day of wave, dominant or subordinate]) or EI ($E_2 < 50$ ng/ml; ultrasonographic data).

cDNA Probes

The bovine P450 17α-hydroxylase full length (509 base pair) cDNA used for hybridization was a generous gift from Dr. M.Waterman (Univ. of Texas, Dallas; Zuber *et al.*, 1986). This probe has been shown to hybridize to RNA obtained from theca but not from granulosa cells of bovine preovulatory follicles before the LH surge (Voss and Fortune, 1993b; Hinshelwood *et al.*, 1993). The 486 base pair bovine P450 Aromatase cDNA was provided by Dr. M. Hinshelwood (Univ. of Texas, Dallas; Hinshelwood *et al.*, 1993) and shown to hybridize to RNA obtained from granulosa but not from theca cells of bovine preovulatory follicles before the LH surge (Hinshelwood *et al.*, 1993). To correct for potential unequal loading of the RNA and variations in transfer efficiency, hybridization with 28S rDNA (human [5028 bp], Gonzalez *et al.*,1985; kindly provided by Dr. R. Schmickel [Univ. Pennsylvania, Philadelphia]) was determined. The cDNAs were labeled with [α-^{32}P]deoxy-CTP (ICN Radiochemicals, Irvine, CA) using a Multiprime DNA Labeling System (Amersham Int., UK; Appendix 5). The labeled cDNA probes were separated from unincorporated label using a Sephadex® G-50 gel filtration column (Pharmacia Biotechnology Inc., Piscataway, NJ).

Statistical Analyses

To further characterize the endocrine and molecular aspects of dominance and atresia in first wave bovine follicles, data presented here and in Chapter 3 were combined and analyzed by correlation analysis, Multiple Analysis of Variance (MANOVA; SAS®, 1988), and Stepwise Regression (STEPWISE; SAS®, 1988). The data used in these analyses included FF concentrations of P_4, A_4, E_2 and mRNA levels for the enzymes P450 SCC, P450-3βHSD, P450 17α-HYD and P450 ARO in follicle walls. These analyses were conducted by two different approaches. In the first approach, steroids in FF were expressed as concentrations per ml and the mRNA levels in arbitrary units per 5μg/total RNA (steady-state levels). In the second approach, the analysis took into account the volume and mass of each follicle (total follicle). Whereas the MANOVA analysis included variability associated with the experimental treatments in the statistical model, the STEPWISE analysis did not. Furthermore, the standardized regression coefficients (Ostle, 1963; SAS®, 1991) obtained from the stepwise regression analysis represent estimates that would be obtained if all variables in the model were standardized to zero mean and unit of variance and are not affected by the scales of measurement of the various model variables. They are computed by dividing a parameter estimate by the ratio of the sample standard deviation of the dependent variable to the sample standard deviation of the regressor. Only independent variables with a probability value ≤ 0.15, were retained in the model (interactive process of stepwise regression; SAS®, 1988).

Results

Estradiol Concentrations in Plasma

An experimental group by day of cycle interaction was detected for plasma E_2 concentrations ($P<.01$; Fig. 4-1, left). A common pattern of plasma E_2 was observed from d 0 to d 8 for all experimental groups of cows. E_2 decreased from 3.29±0.26 pg/ml on the day of estrus (d 0) to 1.46±0.26 pg/ml on d 2. A peak of plasma E_2 was observed at d 5 (2.83±0.35 pg/ml) and then plasma values returned to 1.38±0.51 pg/ml at d 8. However, two patterns of plasma E_2 were observed after d 8 (Fig. 4-1, left). In D12-I cows, plasma E_2 concentrations barely changed until D12, at which time a modest increase was observed ($\bar{x}$ d 9-d 12 = 1.49±0.54 pg/ml). In contrast, D12+I cows had a sharp increase in plasma E_2 concentrations after injection of $PGF_{2\alpha}$ and insertion of a Norgestomet implant on d 8 ($\bar{x}$ d 9-d 12 = 5.47±0.64 pg/ml). In Chapter 3, cows in the D12+I experimental group were divided into two groups based upon plasma progesterone profiles after d 8 of the estrous cycle. One type of profile indicated complete CL regression (P_4=0.83±0.70 ng/ml; n=4; Fig. 3-5, right) whereas the other group had incomplete CL regression (P_4=3.03±0.56 ng/ml; n=7; Fig. 3-5, right). In the D12+I experimental group (Fig. 4-1, right), further analysis showed that four cows of the complete CL regression subgroup had a pronounced increase ($p<.01$) in plasma E_2 concentrations ($\bar{x}$ d 9-d 12 = 8.68±0.91 pg/ml) whereas the other seven cows of the

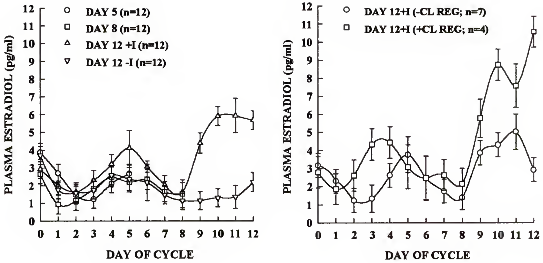

Figure 4-1. Plasma concentrations of E_2 during day 0 to day 12 of synchronized estrous cycles in dairy cows.Data represent least square means (±SEM) of plasma steroid concentrations in cows in four experimental groups (D5, D8, D12-I and D12+I; left) or in one experimental group (D12+I, with and without complete CL regression; right).

incomplete CL regression subgroup did not ($\bar{x}$ d 9-d 12 = 4.02±0.73 pg/ml). Interestingly, the cows that had higher plasma E_2 concentrations between d 9 and d 12 (n=4), exhibited an earlier peak of plasma E_2 around d 3-4 (2 d duration) and had undergone complete corpus luteum (CL) regression after injection of $PGF_{2\alpha}$ and insertion of a progestin implant on d 8 (Fig. 3-5, right). In contrast, cows within the D12+I experimental group that did not undergo CL regression (Fig. 3-5, right), plasma E_2 concentrations were lower between d 9 and d 12 (n=7), and these cows had a one day peak of E_2 that was delayed until d 5.

Androstenedione and Estradiol Concentrations in Follicular Fluid

Follicular fluid A_4 concentrations were different between the experimental groups ($P<.04$; Fig. 4-2, left). The D5 dominant follicles had a high level of FF A_4 which decreased markedly by D8 (135.19±35.83 vs 2.29±33.78 ng/ml, $P<.02$). A major difference was detected between D12+I and D12-I DF (73.66±32.04 vs 6.08±32.04 ng/ml, $P<.03$). Although concentrations of FF E_2 were low and did not change in SF across experimental groups, E_2 concentrations of DF did change between experimental groups (experimental group by follicle interaction, $P<.10$; Fig. 4-2, right). Follicular fluid concentrations of E_2 were higher in D5 DF compared to D8 DF (868.35±268.96 vs 226.44±310.56 ng/ml, $P<.06$) and D12+I DF was greater than D12-I DF (1223.34 vs 33.78±294.63 ng/ml, $P<.01$). Within the D12+I DF experimental group, two distinct follicular populations were observed among cows and was associated with whether CL regression was complete or incomplete (Fig. 4-3). The complete CL regression subgroup (n=4) had higher concentrations of FF A_4 (133.17±29.37 vs 42.23±23.98 ng/ml, $P<.08$) and E_2 (2811.13±252.71 vs 471.89±206.34 ng/ml, $P<.01$) than the incomplete CL regression subgroup of cows (n=7).

Ovarian follicles were classified as either E_2 active ($E_2 \geq 50$ ng/ml; ultrasonographic data [size, day of wave, dominant or subordinate) or E_2 inactive ($E_2 <$ 50 ng/ml; ultrasonographic data). Table 4-1 depicts the frequency of follicles (DF vs SF) within each experimental group (D5, D8, D12-I and D12+I) with FF E_2 < or ≥ 50 ng/ml (experimental group by follicle interaction, $P<.01$). Day 5 and D8 DF, had higher frequency of follicles with $E_2 \geq 50$ ng/ml compared to D12-I and D12+I DF (22/22 vs

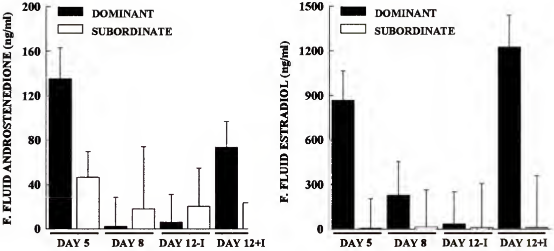

Figure 4-2. Follicular fluid concentrations of A_4 (left) and E_2 (right) in first wave DF and SF of dairy cows.

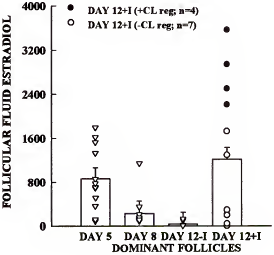

Figure 4-3. Follicular fluid concentrations of E_2 in first wave DF of dairy cows.

Table 4-1. Frequency of follicles (dominant and subordinates) with E_2 concentrations <50 ng/ml or ≥50 ng/ml in the four experimental groups studied (day 5, E_2 active; D8 transitional E_2 active; D12-I atretic and D12+I E_2 active-sustained).

	Day 5[a]		Day 8[b]		Day 12-I[c]		Day 12+I[d]	
E_2 (ng/ml)	<50	≥50	<50	≥50	<50	≥50	<50	≥50
DF[e]	0	12	0	10	9	2	2	9
SF[f]	12	0	10	0	6	5	9	2

[a] Day 5 E_2 active experimental group,
[b] Day 8 transitional E_2 active experimental group,
[c] Day 12-I atretic experimental group,
[d] Day 12+I E_2 active-sustained experimental group,
[e] Dominant follicle,
[f] Subordinate follicle,
E_2 concentrations (ng/ml) orthogonal contrasts:
Experimental group by follicle interaction (P<.01);
DF, Experimental group (P<.01), D5&D8 vs D12-I&D12+I (P<.01), D5 vs D8 (P<.99), D12-I vs D12+I (P<.01);
SF, Experimental group (P<.10), D5&D8 vs D12-I&D12+I (P<.02), D5 vs D8 (P<.99), D12-I vs D12+I (P<.48).

11/22, P<.01). Although the frequencies of DF with E_2 ≥ 50 ng/ml were similar between D5 and D8 DF (12/12 vs 10/10, P<.99), the frequencies of DF with E_2 ≥ 50 ng/ml were strikingly higher in D12+I compared to D12-I DF (9/11 vs 2/11, P<.01). Contrasting results were observed among SF. The frequency of SF with E_2 ≥ 50 ng/ml were similar between D5 and D8 DF (0/12 vs 0/10, P<.99) and between D12-I and D12+I DF (2/11 vs 5/11; P<.49). Therefore, utilizing ultrasonographic information and E_2 concentrations in FF, the majority of D5, D8 and D12+I DF were classified as E_2 active (31/33) and the majority of D12-I DF were classified as E_2 inactive (9/11). Conversely, the majority of D5, D8, D12+I and D12+I SF were classified as E_2 inactive (37/44).

P450-17α-HDY mRNA Contents in Follicle Walls

The Northern blot of RNA extracted from follicle walls of eight representative DF (2 follicles per experimental group) and hybridized with P450 17α-HYD cDNA is depicted in the top of Fig. 4-4 whereas the picture of the ethidium bromide stained gel is depicted in the bottom of Fig. 4-4. P450 17α-HYD hybridization signal was present at 2.0 Kb. No differences in 28S rRNA levels were observed between dominant and subordinate follicles (233.24±19.26 vs 227.13±19.28 AU, $P<.83$; data not shown) nor between experimental groups ($P<.12$); therefore, P450 17α-HYD mRNA levels in each follicle wall was adjusted for 28S rRNA levels to account for potential differences in unequal loading and transfer efficiency.

The RNA dot blot analysis of P450 17α-HYD mRNA levels of a representative set of follicles is depicted in Fig 4-5. Cows during the second experimental period (unilateral ovariectomized) tended to have increased steady state levels of P450 17α-HYD mRNA compared to cows during the first experimental period (57.73±3.40 vs 50.20±3.31, $P<.13$; Fig 4-6, left). Steady state levels of P450 17α-HYD mRNA in SF did not change across experimental groups, but did change for DF (experimental group by follicle interaction, $P<.08$; Fig. 4-6; right). When DF were analyzed separately, D5 and D8 DF had higher levels of P450 17α-HYD mRNA compared to D12-I and D12+I DF (67.25±7.91 vs 51.55±8.11 AU, $P<.07$). Additional orthogonal comparisons indicated that D5 DF tended to have higher levels of P450 17α-HYD mRNA compared to D8 DF

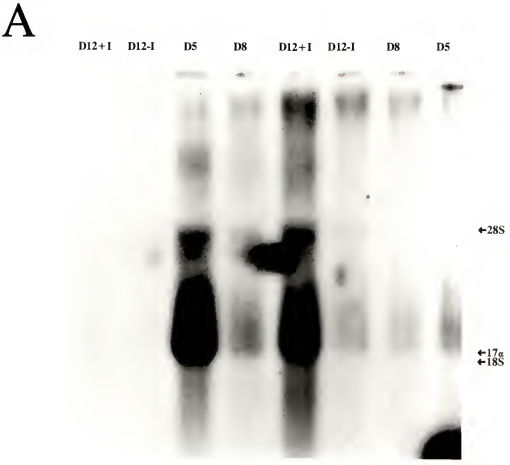

Figure 4-4. Northern blot analysis of P450 17α-HYD. Ten μg of total RNA extracted from first wave DF at D5, D8, D12-I and D12+I were loaded per lane. A. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 17α-HYD cDNA. The hybridization signal was present at 2Kb. B. Picture of ethidium bromide stained gel demostrating equal loading of RNA in each lane.

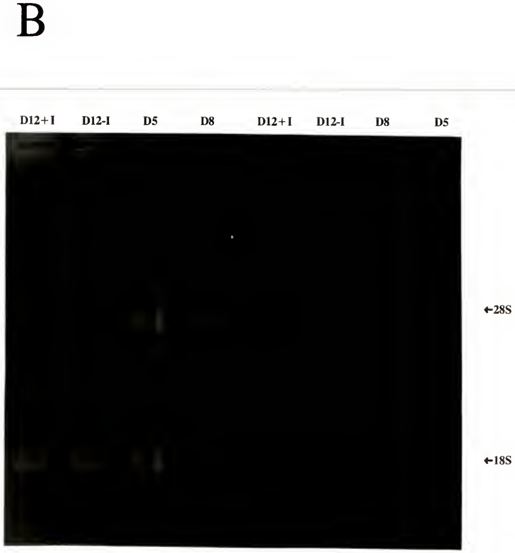

Figure 4-4. continued

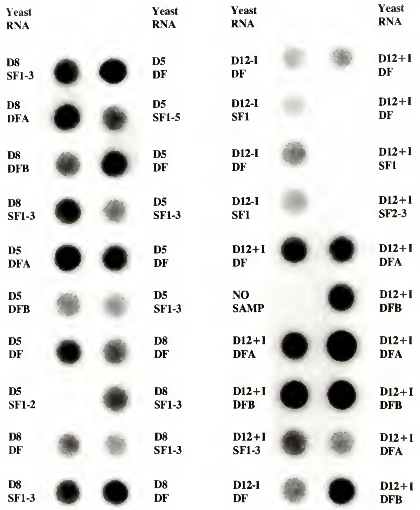

Figure 4-5. Dot blot analysis of P450 17α-HYD. Five μg of total RNA extracted from first wave DF or SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 17α-HYD cDNA.

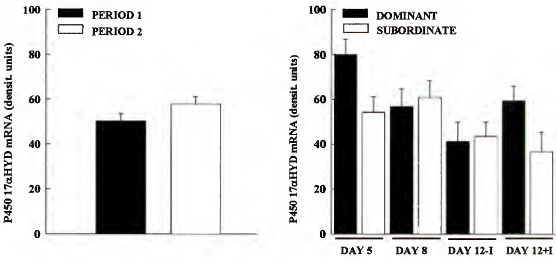

Figure 4-6. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 17α-HYD cDNA to mRNA from first wave DF and SF of non-lactating dairy cows. Data represent least square means (±SEM) of hybridization intensity in cows in two experimental groups (period 1 and 2; left) or in four experimental groups and two follicle types (D5, D8, D12-I and D12+I; dominant and subordinate; right) .

(45.13±9.47 vs 57.98±6.75 AU, $P<.30$). The P450 17α-HYD mRNA levels decreased in a linear fashion in the process of atresia of the dominant follicle (P450 17α-HYD mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF > D12-I atretic DF; $Y=104.72+[-5.45X]$; $R^2=0.96$). However, when D12+I DF (E_2 active sustained) rather than D12-I DF (atretic) were included in the regression analysis, a substantial reduction in the slope of the equation was observed (P450 17α-HYD mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF ≤ D12-I atretic DF; $Y=88.32+[-2.76X]$; $R^2=0.60$). Subordinate follicles P450 17α-HYD mRNA levels did not differ across experimental groups ($P<.15$).

P450 ARO mRNA Contents in Follicle Walls

A Northern blot of RNA from eight representative DF probed for P450 ARO mRNA is shown in the top of Fig. 4-7. The hybridization signal for P450 ARO was present at 6.0 Kb. The picture of the ethidium bromide stained gel demonstrating equal intensity of the 28S and 18S rRNA bands across the lanes is presented in the bottom of Fig. 4-7.

Preliminary analysis of dot blots indicated that the four experimental groups did not differ in the 28S rRNA levels ($P<.48$); therefore, P450 ARO mRNA levle in each follicle was adjusted for its corresponding 28 S rRNA level. The RNA dot blot analysis of P450 ARO mRNA for a representative set of follicles is depicted in Fig 4-8.

The ovariectomized cows had slightly higher steady state levels of P450 ARO mRNA than intact cows ($P<.06$; Fig. 4.9, left). Levels of P450 ARO mRNA did not follow the same pattern in DF and SF across the four experimental groups (experimental group by follicle interaction, $P<.03$; Fig 4-9). Analysis of P450 ARO mRNA levels in DF detected an experimental group effect ($P<.01$). D5 and D8 DF had higher P450 ARO mRNA abundance compared to D12-I and D12+I counterparts (55.56 ± 8.06 vs 23.06 ± 8.38 AU, $P<.01$). However, no differences between means of D5 and D8 DF (61.89 ± 7.51 vs 49.24 ± 8.61 AU, $P<.28$) nor D12-I and D12+I DF (15.64 ± 9.28 vs 30.49 ± 7.49 AU, $P<.25$) were detected. The P450 ARO mRNA levels decreased in a linear fashion during the process of atresia of the dominant follicle (P450 ARO mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF > D12-I atretic DF; $Y=99.85+[-6.93X]$; $R^2=0.96$). However, when D12+I DF (E_2 active sustained) rather

A

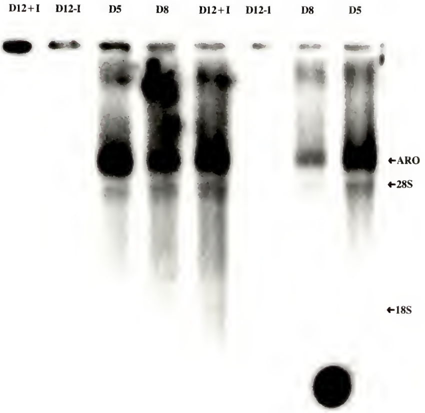

Figure 4-7. Northern blot analysis of P450 ARO. Ten μg of total RNA extracted from first wave DF at D5, D8, D12-I and D12+I were loaded per lane. A. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 ARO cDNA fragment (486 bp). The hybridization signal was present at 6 Kb. B. Picture of ethidium bromide stained gel demostrating equal loading of RNA in each lane.

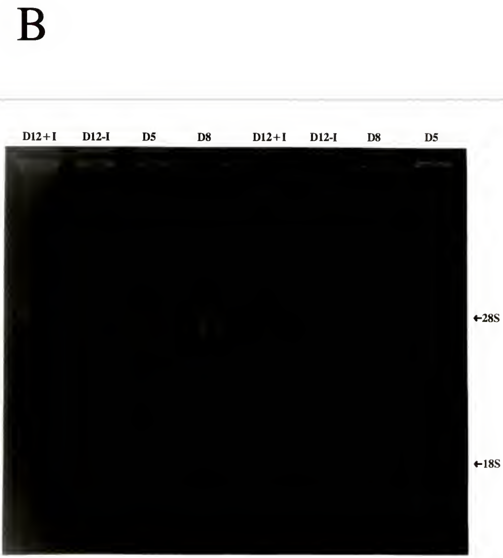

Figure 4-7. continued

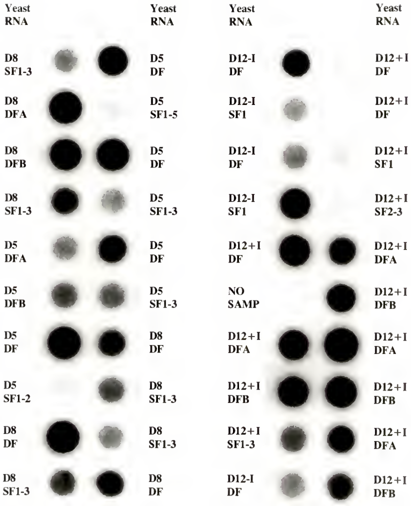

Figure 4-8. Dot blot analysis of P450 ARO. Five μg of total RNA extracted from first wave DF or SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled P450 Aromatase cDNA fragment (486 bp).

than D12-I DF (atretic) were included in the regression analysis, a substantial reduction in the slope of the equation was observed (P450 ARO mRNA levels in D5 E_2 active DF > D8 transitional E_2 active DF ≤ D12-I atretic DF; $Y=84.77+[-4.46X]$; $R^2=0.99$). No experimental group effect or difference among means estimated by orthogonal contrasts were detected for P450 ARO mRNA levels of SF ($P<.39$).

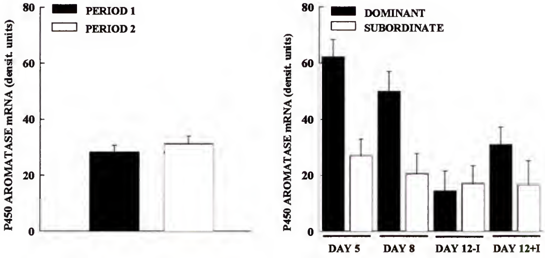

Figure 4-9. Least square means (±SEM) of hybridization intensity (arbitrary units) of P450 ARO cDNA to mRNA from first wave DF and SF of non-lactating dairy cows. Data represent least square means (±SEM) of hybridization intensity in cows in two experimental groups (period 1 and 2; left) or in four experimental groups and two follicle types (D5, D8, D12-I and D12+I; dominant and subordinate; right) .

Correlation and Partial Correlation Analyses

Simple (top-right) and partial correlations (bottom-left) between P_4, A_4 and E_2 FF concentrations (ng/ml) and mRNA steady state levels ($5\mu g$ total RNA/follicle [s]) for P450 SCC, P450 3β-HSD, P450 17α-HYD and P450 ARO enzymes in DF are presented in Table 4-2. The following simple positive correlations were found: 1) follicular fluid P_4 with steady state levels of P450 SCC ($r=0.29$, $P=0.05$); 2) follicular fluid E_2 with A_4 ($r=0.54$, $P<0.01$) and with follicle diameter ($r=0.32$, $P=0.02$); 3) P450 17α-HYD with diameter of the follicle ($r=0.31$, $P=0.04$) and with P450 3β-HSD ($r=0.46$, $P<0.01$); 4) P450 SCC with P450 3β-HSD ($r=0.78$, $P<0.01$); 5) P450 ARO with P450 SCC ($r=0.46$, $P<0.01$), P450 3β-HSD ($r=0.68$, $P<0.01$), and with P450 17α-HYD ($r=0.33$, $P=0.02$).

Partial correlations (Table 4-2, bottom-left) were obtained after adjusting for the effects of experimental group, period, experimental group by period and for cow variation. This analytical approach basically shows the follicle to follicle association on a within cow basis and attempts to minimize the marked differences imposed by experimental groups and periods. No changes in the signs of all the partial correlations was observed. The following partial correlations were found significant: 1) FF P_4 was negatively correlated with FF E_2 ($r=-0.51$, $P=0.06$); 2) A_4 was negatively correlated with P450 SCC ($r=-0.57$, $P=0.03$), and with P450 3β-HSD ($r=-0.49$, $P=0.07$); 3) P450 SCC was positively correlated with P450 3β-HSD ($r=0.85$, $P<0.01$) and 4) P450 3β-HSD was positively correlated with P450 ARO ($r=0.56$, $P=0.03$).

Table 4-2. Simple (top right) and partial (bottom left) correlations between P_4, A_4 and E_2 FF concentrations (ng/ml) and steady state mRNA levels (arbitrary units/5μg total RNA/ follicle wall) of P450 SCC, P450 3βHSD, P450 17αHYD and P450 ARO enzymes in dominant follicles. Within parenthesis are the P values for each coefficient.

	P_4	A_4	E_2	DIAM[a]	SCC[b]	3βHSD[c]	17αHYD[d]	ARO[e]
P_4		-0.12 (0.43)	-.18 (0.23)	-0.03 (0.85)	0.29 (0.05)	0.22 (0.14)	-0.01 (0.94)	0.08 (0.57)
A_4	-0.16 (0.57)		0.54 (<0.01)	-0.09 (0.54)	-0.08 (0.57)	-0.07 (0.63)	-0.07 (0.65)	0.004 (0.97)
E_2	-0.51 (0.06)	0.48 (0.07)		0.34 (0.02)	-0.15 (0.32)	-0.19 (0.21)	0.01 (0.91)	-0.25 (0.09)
DIAM	0.18 (0.52)	-0.15 (0.60)	-0.06 (0.83)		-0.10 (0.51)	0.02 (0.86)	0.31 (0.04)	-0.05 (0.73)
SCC	-0.02 (0.94)	-0.57 (0.03)	-0.38 (0.16)	-0.07 (0.78)		0.78 (<0.01)	-0.08 (0.58)	0.46 (<0.01)
3βHSD	-0.08 (0.78)	-0.49 (0.07)	-0.40 (0.14)	-0.03 (0.90)	0.85 (<0.01)		0.46 (<0.01)	0.68 (<0.01)
17αHYD	0.23 (0.42)	0.04 (0.87)	-0.16 (0.56)	-0.02 (0.92)	-0.17 (0.55)	0.28 (0.32)		0.33 (0.02)
ARO	-0.10 (0.72)	-0.46 (0.09)	-0.41 (0.14)	0.13v (0.64)	0.31 (0.26)	0.56 (0.03)	0.26 (0.36)	

[a] Diameter of dominant follicle,
[b] P450 Side Chain Cleavage mRNA levels,
[c] P450 3βHSD mRNA levels,
[d] P450 17αhydroxylase-17,20 lyase mRNA levels,
[e] P450 Aromatase mRNA levels.

Simple and partial correlations also were obtained utilizing steroid contents per follicle (ng/follicle) and steroidogenic enzyme mRNA contents per follicle (arbitrary units/total RNA/follicle). These analyses are presented in the top-left of Table 4-3 (simple

Table 4-3. Simple (top right) and partial (bottom left) correlations between P_4, A_4 and E_2 FF contents (ng/follicle) and mRNA contents (arbitrary units/total RNA/follicle wall) of P450 SCC, P450 3βHSD, P450 17αHYD and P450 ARO enzymes in DF. Within parenthesis are the P values for each coefficient.

	P_4	A_4	E_2	SCC[a]	3βHSD[b]	17αHYD[c]	ARO[d]
P_4		0.22 (0.14)	0.17 (0.25)	0.34 (0.02)	0.32 (0.03)	0.32 (0.03)	0.33 (0.03)
A_4	0.41 (0.13)		0.74 (<0.01)	0.16 (0.28)	0.16 (0.30)	0.21 (0.17)	0.17 (0.26)
E_2	-0.35 (0.21)	0.46 (0.09)		0.27 (0.07)	0.26 (0.09)	0.32 (0.03)	0.28 (0.06)
SCC	0.59 (0.02)	0.38 (0.17)	0.002 (0.99)		0.97 (<0.01)	0.96 (<0.01)	0.99 (<0.01)
3βHSD	0.51 (0.05)	0.26 (0.35)	-0.06 (0.81)	0.98 (<0.01)		0.96 (<0.01)	0.97 (<0.01)
17αHYD	0.56 (0.03)	0.29 (0.30)	-0.05 (0.85)	0.97 (<0.01)	0.97 (<0.01)		0.98 (<0.01)
ARO	0.61 (0.01)	0.37 (0.19)	-0.01 (0.95)	0.99 (<0.01)	0.97 (<0.01)	0.98 (<0.01)	

[a] P450 Side Chain Cleavage mRNA content,
[b] P450 3βHSD mRNA content,
[c] P450 17αhydroxylase-17,20 lyase mRNA content,
[d] P450 Aromatase mRNA content.

correlations) and in the bottom-left of Table 4-3 (partial correlations). The following simple correlations were detected as being significant: 1) FF A_4 was positively correlated with E_2 ($r=0.74$, $P<0.01$); 2) FF P_4 was positively correlated with mRNAs for the four steroidogenic enzymes ($r=0.32$ to 0.34, $P=0.02$ to 0.03); 3) FF E_2 was positively correlated with P450 SCC ($r=0.27$, $P=0.07$), P450 3β-HSD ($r=0.26$, $P=0.09$), P450 17α-HYD ($r=0.32$, $P=0.03$) and P450 ARO ($r=0.28$, $P=0.06$) and 4) all four classes of mRNA encoding the steroidogenic enzymes were positively correlated with each other ($r \geq 0.96$, $P < 0.01$).

The partial correlations of FF steroids and steroidogenic enzyme mRNA contents expressed on a per follicle basis are presented in the bottom-left of Table 4-3. At least three salient relationships were observed: 1) The mRNA levels for the four steroidogenic enzymes were all positively correlated with each other ($r \geq 0.97$, $P < 0.01$); 2) FF A_4 and E_2 were correlated positively ($r = 0.46$, $P = 0.09$) and 3) FF P_4 was positively correlated with the four steroidogenic enzymes ($r = 0.51$ to 0.61, $P = 0.01$ to 0.05).

Stepwise Regression Analysis and Standardized Regression Coefficients

Standardized regression coefficients obtained from stepwise regression analyses of FF steroids and mRNA contents of steroidogenic enzymes in whole DF are presented in Figure 4-10. Only SRC with a probability value ≤ 0.15 (stepwise regression) were included in the diagram of Fig. 4-10. The SRC are comparable within a variable but not comparable across variables. For example, there are four arrows (SRC) pointing to P450 3β-HSD mRNA content: P450 SCC (1.45), P450 ARO (-1.09), P450 17α-HYD (0.64) and E_2 (-0.04). Each arrow, has a SRC which indicates the relationship between the independent variable (i.e. P450 SCC mRNA) and the dependent variable (P450 3β-HSD mRNA) and ranks these independent variables based upon the magnitude of the coefficient to the other independent variables in how each relates to the specific dependent variable. For example, a unit change in P450 SCC mRNA content was associated with a 2.2-fold greater increase in P450 3β-HSD mRNA content than did P450 17α-HYD mRNA content

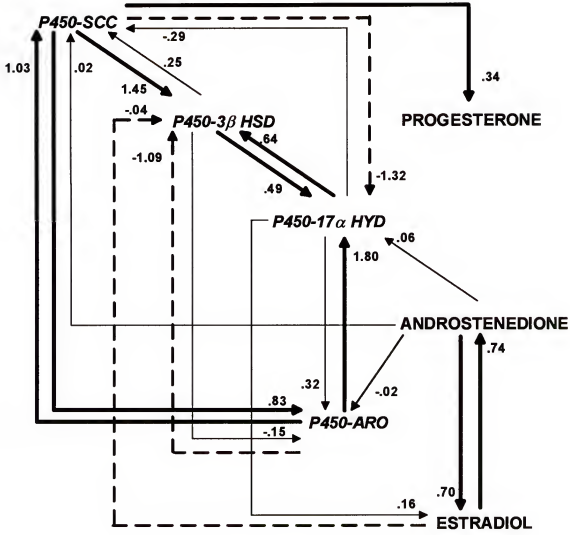

Figure 4-10. Standardized partial coefficients obtained from stepwise regression analyses of follicular fluid steroid concentrations and steroidogenic enzymes mRNA contents in whole dominant follicles.Bolded full arrows represent strong positive relationships whereas bolded dashed arrows represent negative relationships. Thin full arrows represent relationships that were considered relatively less important or not important at all.

(1.45/0.64=2.20). Conversely, a unit change in P450 ARO mRNA content was associated with a decrease in P450 3β-HSD mRNA content that was 27.2-fold greater than the negative association with estradiol content (-1.09/-0.04). However, it is incorrect to state that P450 ARO mRNA content was better correlated with P450 17α-HYD mRNA content (1.80) than with P450 SCC mRNA content (1.03). Some interesting relationships were detected. Positive and strong associations were observed for RNA levels of the steroidogenic enzymes (1.45 from P450 SCC to P450 3β-HSD, 0.49 from P450 3β-HSD to P450 17α-HYD, 0.32 from P450 17α-HYD to P450 ARO, 0.83 from P450 SCC to P450 ARO and 1.03 from P450 ARO to P450 SCC). However, three negative relationships also were observed. E_2 (-0.04) and P450 ARO (-1.09) were negatively correlated with P450 3β-HSD, and P450 SCC (-1.32) was negatively correlated with P450 17α-HYD. The steroids in follicular fluid had three positive correlations. Androstenedione was correlated positively with E_2 (0.70) and vice versa (0.74) and P450 SCC was correlated positively with follicular fluid P_4.

Discussion

In this study, we characterized qualitative and quantitative changes in steady state levels of P450 17α-HYD and P450 ARO mRNA, and follicular fluid A_4 and E_2 concentrations in first wave dominant and subordinate follicles during four very distinct stages of follicular development (D5=E_2 active DF, D8=non-E_2 active DF, D12-I=atretic DF and D12+I=E_2 active sustained DF). The data reported in this chapter support the hypotheses that mRNA gene expression of P450 17α-HYD and P450 ARO decreases in a linear fashion between E_2-active (D5), transitional E_2 active (D8) dominant follicles and atretic dominant follicles (D12-I), and gene expression is maintained or increased in D12+I dominant follicles. However, data reported in this chapter do not support the hypothesis that the observed reduction in androstenedione (A_4) production in FF is paralleled by a reduction in P450 17α-HYD gene expression in transitional E_2 active DF (D8). Furthermore, the standardized regression coefficients obtained from stepwise regression analyses represent a novel approach to study follicle interrelationships in dominant follicles at different stages of dominance and atresia.

Plasma profiles of E_2 were in agreement with previous studies (Sirois and Fortune, 1990; Badinga *et al.,* 1992). The increase in plasma concentrations of E_2 observed on d 5 of the estrous cycle coincides with the time when concentrations of FF E_2 are highest during the first wave of follicle development (Fig. 4-2, right) and also coincides with the period of maximum expression of dominance achieved by the DF (Chapter 3; Fig. 3-2, bottom). The decrease in plasma E_2 concentrations observed after d5 represent the transition towards atresia of these DF. Around d 9-d 11 of the estrous cycle, plasma E_2 concentrations in D12-I cows were two to four-fold lower than those observed at d 5 for all experimental groups. These low E_2 concentrations in plasma are representative of an atretic follicle which is in sharp contrast to those observed in cows of the D12+I experimental group with a sustained active DF. Plasma concentrations of E_2 in cows of D12+I were six times higher than cows of the D12-I experimental group. Cows in the D12+I experimental group, that underwent complete CL regression (n=4) after injection of $PGF_{2\alpha}$ and insertion of a progestin implant on d8, had high plasma concentrations of

E_2 from d 9 to d 12. Cows that had incomplete CL regression (n=7) had lower plasma E_2 concentrations from d 9 to d 12 of the estrous cycle. These latter values were comparable to those observed on d 5 for all experimental groups. Moreover, the peak of plasma E_2 observed in cows with incomplete CL regression on d 5 lasted only one day compared to cows with complete CL regression in which the early diestrous increase in E_2 lasted for two days. When normal luteolysis takes place, E_2 from the DF induces oxytocin receptor formation in the uterine endometrium and oxytocin from the posterior pituitary and/or the CL stimulates secretion of luteolytic pulses of PGF2α from the uterus (McCracken *et al.*, 1984; Flint and Sheldrick, 1986). More recently, Beard and Hunter (1994) demonstrated that E_2 plays a key regulatory role in the control of functional oxytocin receptors and occurrence of premature luteolysis in GnRH-treated anestrous ewes. The extended peak of plasma E_2 observed on d 3 and d 4 of the estrous cycle in cows that underwent complete CL regression in response to injection of $PGF_{2\alpha}$ could have been responsible for increased concentrations of E_2 receptors, which would augment the estrogenic stimulation of oxytocin receptors. Therefore, in the present study, a longer period of E_2 priming during early stages of the estrous cycle may have facilitated the induced luteolysis on d 8 of the estrous cycle in response to exogenous $PGF_{2\alpha}$. However, insufficient E_2 priming during the same period may have caused incomplete luteolysis in some cows. For example, the initial injection of $PGF_{2\alpha}$ induces luteolysis; however, complete luteolysis is dependent upon further secretion of uterine $PGF_{2\alpha}$ through an oxytocin-oxytocin receptor mechanism that is enhanced in animals exposed to an earlier and greater increase in plasma E_2 from the first wave DF. As discussed in Chapter 3 (Fig.

3-6, right), no differences in plasma P_4 were observed between both group of animals during the first 7 days of the estrous cycle. Therefore insufficient P_4 priming was not considered a probable factor contributing to incomplete CL regression (Beard *et al.*, 1991; Beard and Hunter, 1994).

FF concentrations of E_2 in cows at D12+I that had complete CL regression were higher than those observed in follicles from cows with incomplete CL regression. This accounts for the increase in plasma concentrations of E_2 during d 9 to d 12 of the estrous cycle between the two sub-groups. These follicles produced an earlier and longer duration increase of plasma E_2 on d 3 and d 4 of the estrous cycle and lends further support to the observation that a higher estrogenic follicle from the first wave DF facilitated a greater luteolytic effect of $PGF_{2\alpha}$ when given on d 8 of the estrous cycle.

In the present Chapter ovarian follicles were classified as either EA or EI with a single measurement of FF E_2 (EA: ≥ 50 ng/ml; EI: <50 ng/ml) and ultrasonographic information (size, "day of wave", dominant or subordinate) whereas in Chapter 5 classification is based on the ratio between absolute concentrations of FF E_2 and P_4 (EPR; EA: EPR$\geq$1; EI: EPR$<$1). The utilization of a single steroid measurement in follicular fluid (i.e. E_2) and a single low M_r IGBPs (i.e. IGFBP-2, -4 or -5) could be used as potential markers for selection of oocytes after aspiration to be used in *in vitro* maturation and fertilization schemes. The observation that FF P_4 and IGFBPs levels were higher in FF of bovine follicles that contained oocytes with reduced developmental potential and in follicles with oocyte cumulus complexes that failed to develop to the blastocyst stage (Hazeleger *et al.*, 1993) gives support to this view. Hence oocytes aspirated from follicles with high amounts of IGFBPs (either 2, 4 or 5) and low E_2 concentrations (e.g. <50 ng/ml) could be discarded whereas oocytes aspirated from follicles with high E_2 concentrations and low amounts of IGFBPs could be utilized in these schemes. Although quantitation of IGFBPS and E_2 in FF currently requires the use of ligand blots and RIA respectively, the future potential of screening FF with commercial antibodies via enzyme immunoassay (EIA) for IGFBPS and available EIA for E_2 (Bouve *et al.*, 1992; el Jabri, 1991) may speed up processing of FF samples for screening. Furthermore, the use of pumps to perform follicular aspiration *in vivo* or with ovaries obtained from the abattoir would permit fast and individual collection of oocytes and FF from follicles which could be processed promptly.

In agreement with previous studies (Ireland and Roche, 1983; Martin *et al.*, 1991; Badinga *et al.*, 1992), concentrations of A_4 and E_2 in the first wave DF were higher at D5 than at D8 and D12-I. Concentrations of FF A_4 in D8 DF decreased 96% from concentrations of A_4 in D5 DF whereas concentrations of FF E_2 decreased 74% from D5 to D8 DF. Conversely, D12+I DF had a 106% increase in FF concentrations of A_4 and a 470% increase in FF concentrations of E_2 compared to D12-I DF. Collectively, these data support the idea that androgen availability becomes the limiting step for aromatization and that limited A_4 leads to atresia of the DF at the time when LH pulse frequency declines towards d 8 of the estrous cycle (Badinga *et al.*, 1992; Rahe *et al.*, 1980; Savio *et al.*, 1993). Moreover, induction of luteolysis and the insertion of a progestin implant to increase LH pulse frequency reversed the process of impending atresia of D8 DF. This view is supported by maintenance of FF concentrations of A_4 and E_2 in D12+I DF at a level comparable to the healthy estrogenic follicle at D5.

Total RNA was isolated from follicle walls because it has been extensively documented that theca interna cells are the only source of P450 17α-HYD (Rodgers *et al.*, 1987; Voss and Fortune, 1993b) whereas granulosa cells are the only source of P450 ARO (Hinshelwood *et al.,* 1993; Voss and Fortune, 1993b) in bovine ovarian follicles. Processing the follicle wall allowed for sufficient amounts of total RNA to probe for different RNAs within individual DF and SF (P450 SCC and P450 3β-HSD, Chapter 3; α-bInhibin and β_A-bInhibin, Chapter 5; IGFBP-2, Chapter 6). Northern blot analysis of individual dominant and subordinate follicles detected a single hybridization signal at 2.0 Kb for P450 17α-HYD mRNA (Fig. 4-4) and at 6.0 Kb for P450 ARO mRNA (Fig. 4-7). Rodgers *et al.* (1987) and Voss and Fortune (1993b) reported similar sizes for the 450 17α-HYD mRNA hybridization signal in theca cells from bovine ovarian follicles. Zuber *et al.* (1986), reported similar sizes for P450 17α-HYD mRNA hybridization signal in adrenal tissue. The 6.0 Kb hybridization signal for P450 ARO mRNA reported in the present study is comparable to the 6.0 Kb signal reported by Hinshelwood *et al.* (1993) utilizing the same homologous bovine cDNA probe. However, Voss and Fortune (1993b) reported an hybridization signal for P450 ARO mRNA of 8.0 Kb utilizing a rat cDNA probe. Although there appears to be a single transcript of 6 Kb for P450 ARO in cattle, there are two transcripts of 3.4 and 2.9 Kb described in human tissues (Steinkampf *et al.*, 1987), three transcripts of 2.7, 2.2 and 1.7 Kb reported in rat tissues (Hickey *et al.*, 1988,

1990) and one transcript of 3.4 or 3.0 Kb identified in pig tissues (Guthrie *et al.*, 1994; Ko *et al.*, 1994). The large size of the bovine transcript compared to the human, rat and pig transcripts appears to result from the presence of a long 3-untranslated region (Hinshelwood *et al.*, 1993).

Size of the dominant follicle (Fig. 3-2, top-left) and dominance (difference in size between the dominant and the first subordinate follicle; Fig. 3-2, bottom) were greater during the first 5 days of the second experimental period (unilateral ovariectomized) compared to the first five days of period 1 (intact). Conversely, size of the subordinate follicle was smaller during the first five days of period 2 compared to period 1. Furthermore, steady state levels of P450 17α-HYD and P450 ARO mRNA were higher in unilateral ovariectomized (period 2) cows compared to intact cows (period 1; Fig. 4-6 and 4-9). Therefore, presence of a more dominant follicle during the first 5 days of the cycle in the second period was coupled with higher gene expression of P450 17α-HYD and P450 ARO during the same time. Whereas P450 3β-HSD is necessary for conversion of DHEA to A_4 in theca cells, P450 ARO is necessary for aromatization of A_4 to E_2 in granulosa cells. This hypothesis is further supported by similar increases in levels of P450 SCC (Fig. 3-9, left) and P450 3β-HSD (Fig. 3-12, left) mRNA levels necessary to support steroidogenesis in these follicles and by increases in α-bInhibin mRNA levels necessary to exert dominance by feed-back inhibition at the pituitary level and or at ovarian level (Chapter 5, Fig. 5-12, left). It is noteworthy to mention, that although we were able to detect differences among experimental periods in gene expression of α-bInhibin and of the four steroidogenic enzymes as well as size of the DF, size of the SF and dominance, we were not able to detect similar differences in follicular products of these genes (i.e. FF concentrations of P_4, A_4, E_2 and α-bInhibin). Steroid hormones production is regulated by four different mechanisms (Hanukoglu, 1992): 1) level of steroidogenic enzymes are ultimately determined by transcription, stability and translation of the mRNAs encoding these enzymes; 2) steroidogenic enzyme activity which is determined availability of cofactors or postranslational modification of the enzymes; 3) substrate availability; and 4) tissue growth. Therefore, it is very likely that lack of correlation between gene expression and steroid hormones in FF could be explained alternatively by mechanisms that affect mRNA translation or by mechanisms that affect enzyme activity such as cofactor availability or postranslational modification of steroidogenic enzymes.

In support of our first hypothesis, mRNA levels of P450 17α-HYD and P450 ARO decreased in a linear fashion in association with atresia of DF (D5 > D8 > D12-I). A 41% decrease in P450 17α-HYD message ($Y=104.72+[-5.45X]$; $R^2=0.96$) and a 74% decrease in P450 ARO message ($Y=99.85+[-6.93X]$; $R^2=0.96$) followed atresia of D12-I DF compared to mRNA levels of healthy D5 DF. Although, D12+I DF mRNA levels for both steroidogenic enzymes were 25% and a 100% higher compared to those observed in D12-I DF; overall, mRNA levels of both steroidogenic enzymes were decreased by 24% ($Y=88.32+[-2.76X]$; $R^2=0.60$) and 50% ($Y=84.77+[-4.46X]$; $R^2=0.99$) D12+I DF compared to D5 DF. Therefore, the changes in slope of both regression equations (i.e. P450 17α-HYD: -5.45 [D12-I] to -2.76 [D12+I]; P450 ARO: -6.93 [D12-I] to -4.46 [D12+I]) indicate that gene expression was maintained in D12+I DF compared to D8 DF in agreement with our second hypothesis. Furthermore, mRNA levels in SF for both steroidogenic enzymes did not change across the four experimental groups.

Results of the present study do not support the hypothesis that reduction in A_4 production in FF is paralleled by reduction in P450 17α-HYD gene expression in transitional E_2 active DF (D8). Concentrations of A_4 in FF decreased 96% but mRNA levels for P450 17α-HYD decreased only 25%. Likewise, the mRNA levels for P450 ARO decreased 20% but FF E_2 concentrations decreased 74%. Although in the present study protein and enzyme activity for both steroidogenic enzymes were not measured, it is evident that changes in mRNA levels of P450 17α-HYD and P450 ARO cannot account for all changes in FF concentrations of A_4 and E_2(Tables 4-2, 4-3). Therefore, other mechanisms such as regulation of enzyme activity may likely participate in regulating follicular steroid production as monitored by levels in FF.

Follicle development is regulated primarily by gonadotrophic hormones through endocrine mechanisms (Richards, 1980; Ireland and Roche, 1987; Lucy *et al.,* 1992), but terminal stages of follicle development may also be regulated at the ovarian level by presence of an E_2 active DF through paracrine and autocrine mechanisms (Hillier, 1994; Magoffin and Erickson, 1994; Savio *et al.,* 1993). The primary signal for initiation of follicle development is increased secretion of FSH by the pituitary gland (Hillier, 1994). Furthermore, FSH stimulates proliferation of granulosa cells and induces LH-responsive mechanisms that sustain steroid secretion by the dominant follicle. Several growth factors and cytokines are produced locally within the ovary, some in response to gonadotropins, and may cause a differential regulation of gene expression for steroidogenic enzymes acting in either a paracrine or autocrine fashion (Simpson *et al.,* 1992). There is an increasing body of evidence that the stimulatory effects of FSH on granulosa cell growth and differentiation are augmented by IGF-I (Giudice, 1992; Hammond *et al.*, 1991). In the present study, IGF-I and IGF-II concentrations in FF (Chapter 6, Fig. 6-6.) did not change among experimental groups or follicles during the process of atresia or during sustained dominance of the first wave dominant follicle. However, dramatic changes in absolute amounts and in the proportions of low M_r IGFBPs (2, 4 and 5; Chapter 6; Fig. 6-9, top-left, bottom-left&right) were detected in the same follicles. Absolute amounts of IGFBP-2, 4 and 5 present in FF were inversely related with E_2 and A_4 concentrations in FF and with P450 17α-HYD and P450 ARO mRNA levels in follicle walls (Chapter 6; Fig. 6-9; Fig. 4-2; Fig. 4-6, right; Fig. 4-9, right). Hence, D5 E_2 active DF had low absolute amounts of IGFBP-2, 4 and 5 and had high concentrations of E_2 and A_4 and high steady state levels of mRNA for P450 17α-HYD and P450 ARO. Conversely, D12-I atretic DF had high absolute amounts of IGFBP-2, 4 and 5 and very low concentrations of E_2 and A_4 and low steady state levels of P450 17α-HYD and P450 ARO. Collectively, these results support the concept that biological responses of IGF-I and IGF-II, which are under normal FSH stimulation, are likely to be regulated by the IGF-binding proteins (IGFBPs; Ui *et al.,* 1989), and that IGFBPs bind to IGF-I and IGF-II in FF and selectively negate their tropic activity (Ui *et al.,* 1989; Magoffin and Erickson, 1994). Furthermore, low amounts of IGFBP-2, 4 and 5 would augment concentrations of free IGF-I and IGF-II in FF and therefore potentially stimulate aromatase activity (Adashi *et* *al.*, 1985a,b; Bergh *et al.*,1991; Christman *et al.*, 1991; Erickson *et al.*,1989,1990; Veldhuis *et al.*,1985,1986,1987), P450 ARO mRNA expression (Steinkamp *et al.*, 1988), and LH receptor synthesis in granulosa cells (Adashi *et al.*,1985c), and androgen biosynthesis in theca cells (Hernandez *et al.*, 1988).

The secondary FSH surge occurring on days -1 and 0 of the estrous cycle (Adams *et al.*, 1992) recruits a cohort of ovarian follicles into the potential ovulatory pool and is responsible for initiation of expression for inhibin subunit mRNA levels during early stages of folliculogenesis (D'Agostino *et al.*,1991). Inhibin produced by granulosa cells exerts a potent and selective stimulation of androgen synthesis in cultured theca cells (Hillier *et al.*, 1991a, 1991b; Hsueh *et al.*, 1987) and in local regulation of folliculogenesis (Woodruff *et al.*, 1990). However, the results reported in this study do not support a strong role of inhibins in stimulating production of androgens. A sharp drop in A_4 concentrations already was detected in D8 DF (Fig. 4-2, left). However, a 50% reduction in total absolute $_{IMB}$bINH and a 20% reduction in the 196-116 form of inhibin were only observed by d12 in D12-I atretic follicles (Chapter 5; Fig. 5-4 and 5-5). In addition, P450 17α-HYD mRNA levels (Fig. 4-6) did not decrease at the same time as FF A_4 concentrations, suggesting that P450 17α-HYD enzyme activity and not message abundance, was the rate limiting step.

Whereas simple correlations coefficients obtained from correlation analysis and standardized regression coefficients obtained from stepwise regression analysis are coefficients unadjusted for animal, treatment, period and the corresponding interactions, the partial correlation coefficients obtained from the multiple analysis of variance are adjusted for sources of variation included in the model. Therefore, simple correlations represent relationships that may be affected by hormonal treatment (i.e. D12+I *vs* D12-I) and other experimental effects. An examination of both types of interrelationships is a useful tool for identifying potential biological relationships between all variables studied. This can be illustrated with the following example: the simple correlation coefficient between FF E_2 and FF P_4 is -0.18 (P=0.23); however, the partial correlation coefficient was -0.51 (P=0.06). To further illustrate these different associations, consider the simple and partial correlations between steady states mRNA levels of P450 SCC, P450 3β-HSD, P450 AROA and FF A_4 concentrations. The simple correlations were -0.08 (P=0.57), -0.07 (P=0.63) and 0.004 (P=0.97) respectively whereas partial correlations were -0.57 (P=0.03), -0.49 (P=0.07) and -0.46 (P=0.09) respectively. Thus the partial correlations indicate strong associations from cow to cow pooled within experimental groups and periods. These strong associations appear to exist regardless of the marked differences that were induced by experimental treatments and provide further evidence to support causal relationships. The other analytical approach that we followed was to adjust steady state levels of mRNA and the steroids concentrations in FF by the mass and volume of each individual follicle. This approach was undertaken to examine associations that could affect the whole animal at the different stages of follicle development. Dominant follicles at different stages of follicle development have different diameter as measured by ultrasonography and likely produce different contents of FF E_2 associated with size differences. Ignoring the volume of the follicle could either underestimate or overestimate endocrine, paracrine or autocrine effects of estradiol on other factors under consideration. The magnitude of the effect of this adjustment can be appreciated with the following example: D5 DF (14 mm diam.) E_2 concentrations (900 ng/ml) are 25% lower than D12+I DF (20 mm) E_2 concentrations (1200 ng/ml) and 300% higher than D12-I DF (15 mm) E_2 concentrations (300 ng/ml). However, when the same data is expressed in reference to the whole follicle, these relationships change quite dramatically: D5 DF E_2 content (1292 ng/follicle) are 75% lower than D12+I DF E_2 content (5026 ng/follicle) and 244% higher than D12-I DF E_2 contents (528 ng/follicle). Therefore, content of E_2 in a D12+I DF outputs is 3.9 times greater than that from a D5 DF and 9.5 times higher than that of a D12-I atretic DF. Simple and partial correlations obtained with this approach differed substantially with those estimated without adjusting for follicle mass and/or volume. The following trends were observed: 1) simple and partial correlation between mRNAs of steroidogenic enzymes had high coefficients and *P* values, 2) simple and partial correlation between FF P_4, E_2 and the steroidogenic enzymes had higher coefficients and higher *P* values, 3) partial correlation between FF E_2 and the steroidogenic enzymes had non-significant *P* values.

FF steroid contents and mRNA content of steroidogenic enzymes in whole DF also were analyzed by stepwise regression analysis to generate SPC to rank relationships between variables of interest. Each coefficient indicates the number of standard deviation changes in the dependent variable associated with a standard deviation change in the independent variable, holding constant all other independent variables. Therefore, these coefficients are comparable within a variable but not across variables. Although they reflect degrees of association they do not necessarily reflect a cause-effect relationship. Interesting relationships and/or degrees of association were established. As expected, FF A_4 and E_2 contents were associated closely. The mRNA levels of P450 ARO, which is the terminal enzyme in the synthesis of E_2, were positively associated with mRNA levels of P450 SCC an enzyme that is the initial rate limiting enzyme of steroid biosynthesis. A strong association was also observed between P450 ARO and P450 17α-HYD supporting coordinated gene expression of an active DF to produce substrate for aromatization (Voss and Fortune, 1993b). Negative associations with potential regulatory importance also were detected. Follicular fluid E_2 concentrations had a slight negative association with P450 3β-HSD mRNA levels. Fortune and Hansel (1979) suggested that high levels of E_2 in FF may suppress P_4 production and provide additional pregnenolone substrate for conversion to androgens via the Δ^5-pathway. This is further supported by the negative association between P450 ARO and P450 3β-HSD mRNA levels (i.e. higher levels of P450 ARO mRNA were associated with lower levels of P450 3β-HSD mRNA). Enhanced levels of P450 SCC mRNA in follicle walls was associated in a positive manner with P_4 contents in FF which are likely related to with the process of atresia in D12-I DF. Messenger RNA levels of P450 SCC were associated negatively with P450 17α-HYD mRNA levels. These coordinated associations probably reflect the process of luteinization and atresia in granulosa cells coupled with a decrease in A_4 precursors for aromatization in theca cells of D12-I atretic DF. The experimental approach used in the present study with follicular walls does not allow for a distinction in gene expression between granulosa and theca cells.

Simple correlations between bovine FF steroid concentrations and mRNA levels of steroidogenic enzymes have been reported in follicles during preovulatory maturation in pigs (Guthrie *et al.,* 1994) but have not been reported for the bovine first wave DF, preovulatory follicles or follicles obtained from the abattoir. We report a thorough analytical approach to study interrelationships of FF steroids and steroidogenic mRNA contents in first wave DF. These series of statistical analyses represent a powerful analytical approach to establish potential relationships and/or degree of associations between several follicular responses. However, these associations should be viewed cautiously since they do not represent and prove direct cause-effects relationships, but provide the basis for design of future experiments to prove a causal biological relationship.

In summary the following conclusions can be drawn from the present study: 1) the majority of D5, D8 and D12+I DF were E_2 active (FF $E_2 > 50$ ng/ml; ultrasonographic information: size, day of wave, dominant or subordinate) whereas the majority of D12-I DF and SF were E_2 inactive or atretic (FF $E_2 < 50$ ng/ml; ultrasonographic information: size, day of wave, dominant or subordinate), 2) steady state levels of P450 17α-HYD and P450 ARO mRNA decreased in a linear fashion during the transition from E_2 active (D5) to transitional E_2 active (D8) dominant follicle and decreased further with atresia of the DF (D12-I), 3) gene expression of both steroidogenic enzymes was maintained or increased under experimental conditions in which dominance was sustained by enhancing LH pulse frequency (D12+I), 4) the dramatic reduction of FF A_4 in FF did not parallel similar reduction in P450 17α-HYD gene expression in transitional E_2 active (D8) dominant follicles suggesting that regulation of enzyme activity and not message availability may the limiting step in A_4 production, and 5) gene expression of P450 ARO was closely associated with P450 SCC and P450 17α-HYD and negatively associated with P450 3β-HSD supporting the commitment of the DF to produce substrate for aromatization.

CHAPTER 5

CONCENTRATIONS OF INHIBIN AND INHIBIN-α SUBUNITS AND mRNA LEVELS OF INHIBIN α- AND β_A SUBUNITS IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLES

Introduction

Inhibins are a family of heterodimeric glycoprotein hormones ($\alpha\beta_A$, $\alpha\beta_B$) produced by granulosa cells of ovarian follicles (Knight *et al.*,1990; Torney *et al.*,1989). In contrast to the situation in rats where inhibin precursors are proteolytically processed within the granulosa cells and the 30 kDa mature form is secreted into follicular fluid (Bicsak *et al.*, 1988), cattle secrete inhibin into the FF as unprocessed dimerized precursors of α (49 kDa) and $\beta_{A,B}$ (58 kDa) subunits (Sugino *et al.*, 1992). The 105 kDa forms first secreted into FF are then post-translationally processed sequentially to smaller 95 and 58 kDa dimeric forms, and then to the mature 31 kDa form (Sugino *et al.*, 1992). The extracellular processing of the pro-α_N-α_C subunit gives rise to a 6 kDa pro sequence and to a 24 kDa α_N peptide (1-166) and a 20 kDa α_C peptide (167-300; Robertson *et al.*, 1989) whereas processing of the pre-pro-$\beta_{A,\ B}$ subunit yields a pro peptide of 43 kDa and a 15 kDa $\beta_{A,\ B}$ subunit (Robertson *et al.*, 1992). The 58 kDa inhibin is formed of a α_N-α_C subunit (43 kDa) joined by disulfide bonds to a $\beta_{A,\ B}$ subunit (15 kDa) whereas the 31 kDa mature inhibin is comprised of a α_C subunit (20 kDa) joined by disulfide bonds to a $\beta_{A,}$

$_B$ subunit (15 kDa; Robertson *et al.,* 1985; Forage *et al.,* 1986; Robertson *et al.,* 1989; Robertson *et al.,* 1992).

In the past, several molecular weight forms of dimeric inhibin (116, Knight *et al.,*1989; 120 kDa, Miyamoto *et al.,*1986; 105 kDa Sugino *et al.,*1989; 58 kDa, 31 kDa; Robertson *et al.,*1986, 1989) as well as monomeric α-inhibin precursor proteins (Robertson *et al.,*1989), and large amounts of monomeric α-inhibin (Knight *et al.,*1989; Robertson *et al.,*1989) and monomeric β_A-Inhibin (Robertson *et al.,*1992) have been isolated from bovine FF. FSH secretion from the pituitary is suppressed by all dimeric forms of inhibin (Miyamoto *et al.,*1986; Robertson *et al.,*1989; Sugino *et al.,* 1989) whereas free monomeric α-inhibin does not alter FSH secretion (Knight *et al.,* 1989; Robertson *et al.,* 1989). Although these α-inhibin precursor proteins (Robertson *et al.,*1989) and monomeric α-inhibin (Knight *et al.,*1989) do not exert any endocrine feed-back at the pituitary level, recent observations suggest that they have paracrine and autocrine roles at the ovarian level (Findlay *et al.,* 1989; Russell *et al.,* 1994; Schneyer *et al.,* 1991). This is supported by the observation that large monomeric α-subunit inhibin precursors (57 kDa) regulate FSH receptor binding and bioactivity (Schneyer *et al.,* 1991) and have an autocrine or paracrine role in regulation of fertility (Findlay *et al.,* 1989; Russell *et al.,* 1994).

Animals immunized against the α_N (1-166, ewe; Findlay *et al.,* 1989; Russell *et al.,* 1994) or the α_C peptides of α-inhibin subunit (heifers [1-26; Scanlon *et al.,* 1993] [18-30, 63-72, 101-122; Morris *et al.,* 1993]) responded quite differently in terms of FSH and inhibin concentrations in plasma, alteration of ovulatory mechanisms, ovulation rates, pregnancy rates and CL formation. Immunization against α_N (1-166; Findlay *et al.*, 1989; Russell *et al.*, 1994) produced a decrease in circulating inhibin concentrations and an increase in circulating FSH levels as a consequence of reduced negative feedback of inhibin on FSH secretion. The reduction in negative feedback can be ascribed to the binding of antibodies to the α_N portion of the 58 kDa or larger molecular mass forms of circulating inhibin causing either direct immunoneutralization of these forms or a block in cleavage of the α_N subunit by serum proteases to form the 32 kDa form. Although circulating P_4 concentrations and LH secretion (i.e. basal LH, pulse amplitude and frequency and preovulatory surge) were not affected by immunization, fewer oocytes were present in the oviduct 2 days after ovulation, with either no change or an increase in number of CL-like structures on the ovary (Russell *et al.*,1994). Only 50% of the ewes conceived in contrast to the control group where 100% were pregnant (Findlay *et al.*, 1989). Collectively, these data suggest that immunization against the α_N peptide impairs fertility via intraovarian mechanisms leading to failure in oocyte release from the follicle.

Conversely, immunization of heifers against the α_C peptide of inhibin induced changes in follicular dynamics and increased ovulation rate in heifers (Morris *et al.*, 1993; Scanlon *et al.*, 1993). Moreover, Forage *et al.* (1987) and Wrathall *et al.* (1992) reported increases in FSH concentrations and number of CL, with no impairment in follicular development and CL function with immunizations of ewes against α_C compared to immunizations against α_N peptide that impaired ovulation and CL development (Russell *et al.*, 1994). The α-inhibin subunit (58 kDa) has been identified *in vitro* as a putative competitor of FSH for FSH receptor binding (Sato *et al.*, 1982; Schneyer *et al.*, 1991). Therefore, the increase in FSH concentrations and ovulation rate observed in animals immunized against the α_C peptide can be attributed to immunoneutralization of inhibin and to increased binding of FSH to the FSH receptor, thus preventing follicular atresia and increased ovulation rate (Morris *et al.*, 1993). In summary, these results clearly support the traditional endocrine role of inhibins in regulation of FSH secretion and in folliculogenesis, but also support important autocrine and paracrine roles of α-inhibin subunits at the ovarian level.

In cattle, total inhibin immunoreactivity (α-subunits and $\alpha\beta$ dimers) in FF increase in parallel with follicle size, P_4 and E_2 concentrations and DF have higher concentrations of inhibin than do atretic follicles as measured by RIA ($_{RIA}$INH; inhibin α_c^{1-26}gly.tyr [standard and tracer]; rabbit antiserum against α_c^{1-32} pig inhibin [α_c^{1-32} pINH] coupled to BSA; Martin *et al.*, 1991). Guibault *et al.* (1993) concluded that $_{RIA}$INH concentrations (α-subunits and $\alpha\beta$ dimers) were higher during the growing phase than during the regressing phase of development for the first wave DF, and $_{RIA}$INH was correlated positively with E_2:A_4 ratios. However, they reported also that dimeric inhibin concentrations (two-site immunoradiometric assay [IRMA, $_{IRMA}$INH]; sheep antibody against α_c^{1-32}; murine antibody against β_A^{82-114}; donkey anti-sheep capture antibody) were higher in the regressing DF compared to the growing DF, and that dimeric inhibin concentrations were correlated negatively with E_2:A_4 ratios. Moreover, FF $_{IRMA}$INH concentrations were substantially lower than $_{RIA}$INH concentrations, and an inverse relationship was detected between both concentrations in FF. Collectively, these data indicate that $_{RIA}$INH increases during growth and decreases during atresia of non-ovulatory DF whereas $_{IRMA}$INH increases with atresia of the non-ovulatory DF. In summary, the diverse array of monomeric (α-subunit: 49, 20) and dimeric (α-β_A, α-$\beta_{B:}$ 116, 105, 95, 58, 31) forms of inhibin present in FF, and the conflicting results between $_{RIA}$INH (detects monomeric [α-subunit] and dimeric forms) and $_{IRMA}$INH (detects dimeric forms [α-β]) in FF suggest that postranslational processing of inhibin molecules may play an important role in the process of dominance and atresia in addition to *de novo* synthesis of inhibin.

Expression of mRNA levels for inhibin α and β_A subunits in follicles and corpora lutea throughout the estrous cycle and gestation were studied in cattle and sheep by Rodgers *et al.* (1989). In cattle, mRNA levels for inhibin α and β_A subunits were detected by Northern blot analyses in antral follicles but neither was detected in ovarian stroma, which potentially could have contained follicles up to 0.5 mm in diameter, or in corpora lutea of cyclic or pregnant cows. Furthermore, in-situ hybridization studies detected the presence of mRNA for inhibin α-subunit in granulosa cells of antral follicles >0.36 mm in diameter whereas inhibin α-subunit and β_A-subunits mRNA were detected in granulosa and theca cells of antral follicles greater than 0.80 mm (Torney *et al.*, 1989). Moreover, no hybridization was observed in cells of the CL (Torney *et al.*, 1989). In summary, although levels of mRNA for inhibin α and β_A subunits have been measured in antral follicles, no description of the stage of follicle development (i.e. dominant or atretic) nor concentrations of inhibins and steroids in follicular fluid were available.

In summary, a detailed study that measures qualitative and quantitative changes in the monomeric and dimeric forms of inhibin in FF and which also compares mRNA levels for α-inhibin and β_A-inhibin in follicle walls of first wave DF and SF at different stages of development in cattle is lacking. The objective of this chapter was to measure in first wave dominant and subordinate follicles at different stages of development of cattle the qualitative and quantitative changes in the different forms of inhibin and inhibin α-subunits in follicular fluid and mRNA levels for α-inhibin and β_A-inhibin. This characterization will document interrelationships between the different forms of inhibin and inhibin α-subunits in follicular fluid and mRNA levels for α-inhibin and β_A-inhibin in estrogen active, transitional estrogen active, atretic and estrogen active-sustained dominant follicles to examine their involvement in dominance and atresia.

Material and Methods

The reader is referred to the material and methods section of Chapter 3 for a detailed description of the experimental animals and reproductive management, ultrasonographic examination and blood collection, tissue collection and handling, steroid hormone RIA in FF, RNA isolation, Northern and dot blot analyses, phosphor screen autoradiography and statitistical analyses utilized in the present section.

Inhibin Assay in Follicular Fluid

Follicular fluid concentrations of inhibins ($\alpha\beta$ dimers and free α-subunits; $_{RIA}$INH) were measured in individual follicles (DF, n=43; SF, n=60) by RIA as described by Ireland *et al.* (1994). These RIA were done in cooperation with Dr. J. Ireland at Michigan

State University. The inhibin RIA was performed using ^{125}I-inhibin as tracer and recombinant inhibin as standard. A mink antiserum (against $\alpha_c^{1\text{-}26}$ inhibin conjugated to human α-globulin) which cross reacts with α-subunits and $\alpha\beta$ forms of inhibin was the antibody used for the RIA. The mink antiserum did not cross-react with human recombinant follistatin, human transforming growth factor β-1, GnRH, fibroblast growth factor, FSH, LH, BSA, bovine gamma globulins, bovine immunoglobulins, bovine α-macroglobulins or human α-globulins (Ireland *et al.*, 1994). The intra- and inter-assay coefficients of variation were $<12\%$. Since this RIA uses an antibody against $\alpha_c^{1\text{-}26}$ polypeptide of α-subunits of inhibin, it measures concentrations of $\alpha\beta$ dimers and free α-subunits of inhibin in FF.

Immunoblot Analysis of Inhibin in Follicular Fluid

Amounts of different forms (M_r) of inhibin ($\alpha\beta$) and inhibin α-subunits were measured in follicular fluid of individual follicles (DF, n=43; SF, n=62) by immunoblot analysis ($_{IMB}$INH; Ireland *et al.*, 1994). These $_{IMB}$INH were done in cooperation with Dr. J. Ireland at Michigan State University. Ten μg of FF proteins/follicle/gel lane were mixed (1:4, vol:vol) in buffer containing 5% SDS, heated at 80°C for 20 min and then subjected to 12% SDS polyacrylamide gel electrophoresis (Laemmli, 1970; Mini-Protein II Vertical Electrophoresis Cell, Bio-Rad, CA) at 200 volts for 45 min under non-reducing conditions. Proteins were transferred from gels to Immobilon P membranes (polyvinylidine difluoride, PVDF; Millipore Corp., MA; Matsudaria, 1987) with 10 mM 3-[cyclo-hexylamino]l-polyvinylidene difluoride, 10% methanol) using a Mini Protean II

Trans-Blot Cell (Bio-Rad, CA). Membranes were blocked with 0.01% non-fat dry milk in TBS (50 mM Tris, 0.5 M NaCl, 0.02% NaN_3) at room temperature (RT) for 2 hrs, and washed 5 times with TTBS (0.05% Tween 20, TBS) for 10 min. Then the membranes were incubated overnight at RT with mink antiserum against inhibin α_c^{1-26}gly.tyr (1:1000 in TTBS). The membranes were washed 7 times for 20 min with TTBS and incubated overnight at RT with radiolabeled ^{125}I-inhibin α_c^{1-26}gly.tyr (^{125}I-INH, 1x10^6cpm/ml, total volume=20 ml; 0.25ml/cm^2). After incubation, the membranes were washed 7 times for 20 min with TTBS (0.7 M NaCl). Broad-range molecular mass protein standards (SDS-PAGE [14-200 KD]; Bio-Rad, CA) were used to determine the size of the each inhibin band in the gels.

cDNA Probes

The cDNA that corresponds to the pro-α_C region of bovine α-Inhibin subunit (450 bp; Forage *et al.,* 1986) and the cDNA that corresponds to the bovine β_A-Inhibin (600 bp; Forage *et al.,* 1986) were generous gifts from Dr. A. Stewart (Biotech Australia PTY. Ltd). Although in-situ hybridization studies have detected the presence of mRNA for α-inhibin subunit in granulosa and theca cells of the bovine ovary, the presence of β_A-inhibin subunit mRNA was restricted to granulosa cells (Torney *et al.,* 1989). *In-vitro* studies have shown that granulosa cells are the major production site of inhibin in the ovary (Erickson and Hsueh, 1978; Ying, 1988). Hybridization with 28S rRNA (human [5028 bp], Gonzalez *et al.*,1985; kindly provided by Dr. R. Schmickel) were determined in Northern and Dot blot filters to correct for potential unequal loading of the RNA and variations in transfer efficiency. The cDNAs were labeled with [α-^{32}P]deoxy-CTP (ICN

Radiochemicals, Irvine, CA) by use of the Multiprime DNA Labeling System (Amersham Int., UK; Appendix 5). The labeled cDNA probes were separated from unincorporated label using a Sephadex® G-50 gel filtration column (Pharmacia Biotechnology Inc., Piscataway, NJ).

Densitometry Analysis

The $_{IMB}$INH membranes were exposed to Kodak X-OMAT AR film with Cronex intensifying screens (Du Pont, Wilmington, DE) at -80° C for 8 days. Densitometric units for each inhibin band in the autoradiograms were determined using a Bio-Rad imaging densitometer model GS-670 and a Molecular Analyst Computer program (Bio-Rad, Hercules, CA), and density of each molecular mass form of inhibin was expressed as arbitrary units (AU) per 10 μg of electrophoresed protein.

Results

Progesterone, Androstenedione and Estradiol Concentrations in Follicular Fluid

The EPR was high in E_2 active (D5) and E_2 active-sustained (D12+I) DF and low in atretic (D12-I) DF. Conversely, EPR ratio was low and did not change in SF across all experimental groups (experimental group by follicle interaction, $P<.05$; Fig. 5-1, left). Among DF, the EPR was different across all experimental groups ($P<.02$). Day 5 E_2 active follicles had higher EPR than D8 E_2 active transitional follicles (22.07 ± 5.10 vs 9.01 ± 5.89, $P<.05$; Fig. 5-1, left), and D12+I DF had higher EPR than D12-I DF (22.77 ± 5.59 vs 0.57 ± 5.59, $P<.02$; Fig. 5-1, left). Conversely, SF of all experimental groups did not have different EPR (D5=0.27 ± 0.08, D8=0.23 ± 0.21, D12-

I=0.43±0.13, D12+I=0.18±0.15; P<.65). The EAR was very high in transitional E_2 active (D8) DF, intermediate in E_2 active (D5) and E_2 active-sustained (D12+I) DF and low in atretic (D12-I) DF. In contrast, EAR was low and did not change in SF across all experimental groups (experimental group by follicle interaction, P<.01; Fig. 5-1, right).

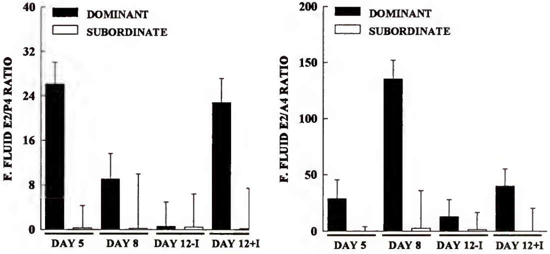

Figure 5-1. Follicular fluid E_2:P_4 ratio (EPR, left) and E_2:A_4 ratio (EAR, right) in first wave DF and SF of dairy cows.

A separate analysis that included only DF, indicated that EAR was significantly augmented in D8 compared to D5 DF (135.25±22.90 vs 28.67±22.90, P<.04; Fig. 5-1, right) and D12+I had higher EAR than D12-I DF (39.94±20.49 vs 12.89±20.49, P<.07; Fig. 5-1, right). No differences in EAR were detected in SF (P<.23; Fig. 5-1, right). Table 5-1 shows the frequency of follicles (DF vs SF) within each experimental group (D5, D8, D12-I and D12+I) with EPR and EAR ≥1 or <1 (experimental group by follicle interaction; EPR, P<.01; EAR, P<.01; Table 5-1). Dominant follicles had a higher frequency of follicles with EPR and EAR ≥ 1 than did SF ([EPR, DF:33/44 vs SF:7/44; P<.01, Table 5-1] [EAR, DF:37/44 vs SF:12/44; P<.01, Table 5-1]. The frequency of DF with EPR ≥1 in D5 (12/12) and D8 (9/10) DF was higher (P<.01; Table 5-1) than the frequency of DF with EPR ≥1 in D12-I (2/11) and D12+I (10/11). Moreover, no differences were observed between D5 and D8 DF (12/12 vs 9/10, P<.54; Table 5-1). However, very striking frequency differences were observed between D12-I and D12+I (2/11 vs 10/11, P<.01; table 5-1). The same trend was observed with the EAR in DF, albeit with one exception. The frequency of follicles with EAR ≥1 was 7/11 in D12-I DF compared to the frequency of follicles with an EPR ≥1 which was 2/11 in the same experimental group. Subordinate follicles in D5 (12/12) and D8 (10/10) had a higher frequency of follicles with EPR<1 compared to D12-I (6/11) and D12+I (9/11, P<.02; Table 5-1). No other differences in frequencies of follicles with EPR <1 were observed (P<.49). However, the frequency of follicles with EAR<1 was different between D12-I (3/11) and D12+I (9/11) SF (P<.04; Table 5-1).

Table 5-1. Frequency of follicles (dominant and subordinates) with E_2:P_4 ratio and E_2:A_4 ratio <1 or ≥ 1 in the four experimental groups studied (day 5, E_2 active; D8 non-E_2 active; D12-I atretic and D12+I E_2 active-sustained).

	Day 5[a]		Day 8[b]		Day 12-I[c]		Day 12+I[d]	
E_2:P_4 ratio	<1	≥ 1	<1	≥ 1	<1	≥ 1	<1	≥ 1
DF[e]	0	12	1	9	9	2	1	10
SF[f]	12	0	10	0	6	5	9	2
E_2:A_4 ratio	<1	≥ 1	<1	≥ 1	<1	≥ 1	<1	≥ 1
DF	2	10	0	10	4	7	1	10
SF	12	0	8	2	3	8	9	2

[a] Day 5 E_2 active experimental group,
[b] Day 8 non E_2 active experimental group,
[c] Day 12-I atretic experimental group,
[d] Day 12+I E_2 active-sustained experimental group,
[e] Dominant follicle, [f] Subordinate follicle,

E_2:P_4 ratio orthogonal contrasts:

Experimental group by follicle interaction ($P<.01$);

DF, Experimental group ($P<.01$), D5&D8 vs D12-I&D12+I ($P<.01$), D5 vs D8 ($P<.54$), D12-I vs D12+I ($P<.01$);

SF, Experimental group ($P<.10$), D5&D8 vs D12-I&D12+I ($P<.02$), D5 vs D8 ($P<1.00$), D12-I vs D12+I ($P<.49$);

E_2:A_4 ratio orthogonal contrasts:

Experimental group by follicle interaction ($P<.01$);

DF, Experimental group ($P<.35$), D5&D8 vs D12-I&D12+I ($P<.41$), D5 vs D8 ($P<.33$), D12-I vs D12+I ($P<.18$);

SF, Experimental group ($P<.01$), D5&D8 vs D12-I&D12+I ($P<.01$), D5 vs D8 ($P<.34$), D12-I vs D12+I ($P<.04$).

Inhibin Concentrations in Follicular Fluid

Total inhibin radioimmunoactivity ($_{RIA}$INH; α-subunits, α-β dimer) concentrations in FF (ng/mg protein) were higher in DF (n=43) than in SF (n=60; 9.03±0.71 vs 4.91±0.84, $P<.05$; Fig. 5-2). No differences in $_{RIA}$INH were detected between experimental groups ($P<.76$; Fig. 5-2) nor between periods ($P<.30$; data not shown). Separate analyses within DF and SF did not detect differences in $_{RIA}$INH concentrations between experimental groups (DF: $P<.21$; SF: $P<.20$; Fig. 5-2).

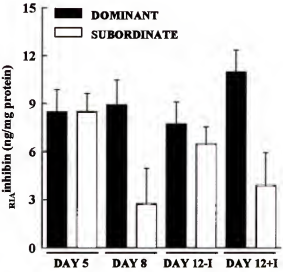

Figure 5-2. Total inhibin radioimmunoactivity ($_{RIA}$INH; α-subunits, α-β dimer) concentrations in follicular fluid (ng/mg protein) in first wave DF and SF of dairy cows

Immunoblot Analysis of Inhibin Content in Follicular Fluid

A representative immunoblot under non-reduced conditions of FF from D5, D8, D12-I and D12+I dominant and subordinate follicles depicts (Fig. 5-3) the diverse array of different molecular forms of inhibins ($_{\text{IMB}}$INH). Forms of inhibin detected by this technique are either monomeric α-subunits or α-β dimers of inhibin. Overall, eight different forms of $_{\text{IMB}}$INH were detected in FF from dominant (n=43) and subordinate (n=62) follicles under these conditions. In decreasing M_r order, these 8 forms were: >300, 196-116, 80, 58, 49, 31, 28 and 25 kDa. However, as the 58 and the 25 kDa forms were present in only 11 and 7 of 105 follicles respectively, they were not included in the statistical analysis of the data presented below. Also it is noteworthy to point out that the 196-116 kDa form is composed of two different forms (196 kDa and 116 kDa) and that the bulk of intensity appears to belong to the 116 kDa form, under the present experimental conditions we were unable to obtain individual densitometric readings for each form.

Total $_{\text{IMB}}$INH intensity (sum of arbitrary densitometric units [AU] for all forms of inhibin for each individual follicle [10 μg of FF protein/follicle]) had different patterns among experimental groups and between DF and SF (experimental group by follicle interaction, $P<.02$; Fig. 5-4). Separate analysis of DF, indicated that D5 and D8 DF had high and similar $_{\text{IMB}}$INH intensities (66.89±7.75 vs 72.82±8.67 AU, $P<.62$; Fig. 5-4) than D12-I and D12+I DF. Day 12+I DF had higher $_{\text{IMB}}$INH intensity than D12-I DF (42.28 vs 28.46±7.75 AU, $P<.07$; Fig. 5-4). SF total $_{\text{IMB}}$INH intensities were not different between experimental groups ($P<.63$).

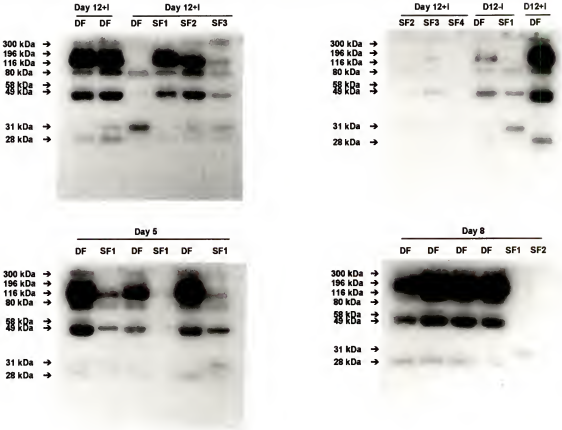

Figure 5-3 Immunoblot analysis (α-subunits, α-β dimers) of non-reduced follicular fluid from representative first wave D5, D8, D12-I and D12+I dominant and subordinate follicles. Ten μg of total protein were electrophoresed per follicle/lane. Membranes were incubated with mink antiserum against inhibin $\alpha_c{}^{1\text{-}26}$gly.tyr, were washed and then were incubated with radiolabeled ^{125}I-inhibin $\alpha_c{}^{1\text{-}26}$gly.tyr (^{125}I-INH). In decreasing M_r order, 8 forms were detected:300, 196-116, 80, 58, 49, 31, 28 and 25 kDa.

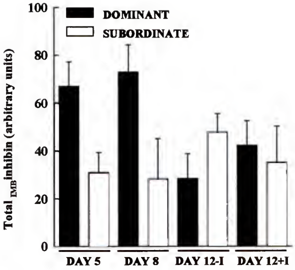

Figure 5-4. Least square means (±SEM) of total $_{IMB}$INH intensity (α-subunits, α-β dimers; sum of arbitrary densitometric units for all forms [300, 196-116, 80, 49, 31 and 28 kDa] of inhibin/follicle) in first wave DF and SF of non-lactating dairy cows.

Four inhibin forms detected in FF (>300, 196-116, 80 and 49 kDa) followed the same pattern of change described for the total $_{IMB}$INH intensity. An experimental group by follicle interaction was detected for all four forms (>300 kDa, $P<.07$; 196-116 kDa, $P<.01$; 80 kDa, $P<.02$; 49 kDa, $P<.01$; Fig. 5-5, top-left, top-right, bottom-left, bottom-right, respectively). Separate analyses for SF $_{IMB}$INH intensities did not detect differences between experimental groups (>300 kDa, $P<.81$; 196-116 kDa, $P<.45$; 80 kDa, $P<.45$; 49 kDa, $P<.24$; Fig. 5-5, top-left, top-right, bottom-left, bottom-right,

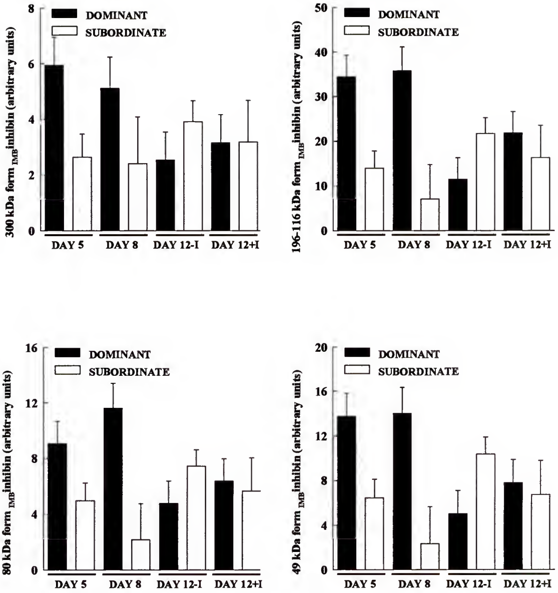

Figure 5-5. Least square means (±SEM) of $_{\mathrm{IMB}}$INH intensity (α-subunits, α-β dimers; arbitrary densitometric units of 300 [top-left], 196-116 [top-right], 80 [bottom-left], 49 [bottom-right] kDa forms of inhibin/follicle) in first wave DF and SF of non-lactating dairy cows. Ten μg of total protein from each DF or SF were loaded per lane. Membranes were incubated with mink antiserum againt inhibin α_c^{1-26}gly.tyr, were washed and then were incubated with radiolabeled ^{125}I-inhibin α_c^{1-26}gly.tyr (^{125}I-INH).

respectively). Conversely, DF $_{IMB}$INH intensities were different across experimental groups (>300 kDa, $P<.02$; 196-116 kDa, $P<.01$; 80 kDa, $P<.01$; 49 kDa, $P<.01$; Fig. 5-5, top-left, top-right, bottom-left, bottom-right, respectively). The D5 and D8 DF $_{IMB}$INH intensities were not different (>300 kDa, $P<.48$; 196-116 kDa, $P<.82$; 80 kDa, $P<.17$; 49 kDa, $P<.92$; Fig. 5-5, top-left, top-right, bottom-left, bottom-right, respectively) whereas $_{IMB}$INH intensity in D12+I DF was greater, albeit sometimes not significant when compared to D12-I DF (>300 kDa, $P<.56$; 196-116 kDa, $P<.07$; 80 kDa, $P<.35$; 49 kDa, $P<.27$; Fig. 5-5, top-left, top-right, bottom-left, bottom-right).

The 31 kDa form of inhibin had an opposite pattern of $_{IMB}$INH intensity compared to the 300, 196-116, 80 and 49 kDa forms, as depicted in Fig. 5-6 (left). $_{IMB}$INH intensity differences were detected between experimental groups ($P<.08$; Fig. 5-6, left). Separate analyses of SF did not detect differences between experimental groups ($P<.50$; Fig. 5-6, left). Conversely, experimental group differences were detected in DF ($P<.07$; Fig. 5-6, left). Similar $_{IMB}$INH intensities were detected in D5 and D8 DF (1.45±0.56 vs 2.65±0.63 AU, $P<.19$; Fig. 5-6, left) whereas higher $_{IMB}$INH intensities were detected in D12-I DF compared to D12+I DF (3.06 vs 1.05±0.56 AU, $P<.03$; Fig. 5-6, left).

Levels of the 28 kDa form of inhibin in FF reflected different patterns of $_{IMB}$INH intensity in DF and SF across experimental groups (experimental group by follicle interaction, $P<.06$; Fig. 5-6, right). Dominant follicles at D5 and D8 had more $_{IMB}$INH intensity than D12-I and D12+I DF (2.97±0.45 vs 1.86±0.43, $P<.03$; Fig. 5-6, right).

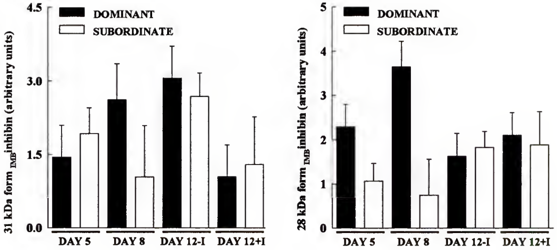

Figure 5-6. Least square means (±SEM) of $_{IMB}$INH intensity (α-subunits, α-β dimers; arbitrary densitometric units of 31 kDa (left) and 28 kDa (right) form of inhibin/follicle) in first wave DF and SF of non-lactating dairy cows.

Although no differences in $_{IMB}$INH intensity were detected between D12-I and D12+I DF (2.10 vs 1.63±0.43, P<.46; Fig. 5-6, right), differences between D5 and d8 DF (2.29±0.43 vs 3.65±0.43, P<.03; Fig. 5-6) were detected. No differences in $_{IMB}$INH intensity of 28 kDa inhibin were found between SF (P<.55; Fig. 5-6, right).

The proportion of $_{IMB}$INH intensity of each inhibin form to the total $_{IMB}$INH in FF for DF are presented in the left panel of Fig. 5-7 whereas those for SF are presented in the right panel of Fig. 5-7. The proportion of $_{IMB}$INH intensity of 300 kDa form was higher in SF compared to DF (13.62±2.11 vs 8.03±1.76 %; P<.06; Fig. 5-7 [left and right]). Dominant follicle differences in % $_{IMB}$INH for the >300 kDa intensity were only present between D12-I and D12+I DF (9.59 vs 7.11±0.91 %; P<.07; Fig. 5-7 [left]). Conversely, in SF D12+I had higher % $_{\mathrm{IMB}}$INH intensity than D12-I SF (24.06±7.36 vs 7.93±3.69 %;P<.07; Fig. 5-7 [right]). The proportion of 196-116 kDa form had a different pattern of percent $_{\mathrm{IMB}}$INH intensity in DF compared to SF (experimental group by follicle interaction, P<.01; Fig. 5-7 [left and right]). The percent $_{\mathrm{IMB}}$INH intensities for D5 and D8 DF were similar (50.97±3.10 vs 50.25±3.47, P<.88 Fig. 5-8 [left]) whereas D12 +I DF had higher $_{\mathrm{IMB}}$INH intensity than D12-I DF (48.55 vs 31.53±3.10, P<.01, Fig. 5-7 [left]). Conversely, in SF, D12-I had higher $_{\mathrm{IMB}}$INH intensity than D12+I DF (43.11±4.06 vs 25.99±8.09, P<.08, Fig. 5-7 [right]). An experimental group by follicle interaction also was detected for the percent $_{\mathrm{IMB}}$INH intensities of the 80 kDa form (p<.03; Fig. 5-7 [left and right]). Separate analyses indicated that the sole source of variation in DF was due to differences in percent $_{\mathrm{IMB}}$INH intensities between D12-I and D12+I DF (19.11 vs 14.92±1.08, P<.02, Fig. 5-7 [left]) whereas no differences in percent $_{\mathrm{IMB}}$INH intensities were found between experimental groups in SF (P<.25, Fig. 5-7 [right]). In addition, no differences in $_{\mathrm{IMB}}$INH intensity for 49 kDa form were detected between experimental groups (P<.39; Fig. 5-7 [left and right]), follicles (P<.57; Fig. 5-7 [left and right]) or their interaction (P<.43; Fig. 5-7 [left and right]). To the contrary, very striking differences in % $_{\mathrm{IMB}}$INH intensities of the 31 kDa form were observed (experimental group by follicle interaction, P<.02; Fig. 5-7 [left and right]). No differences in % $_{\mathrm{IMB}}$INH intensity were present in SF (P<.60; Fig. 5-7 [right]); however, a large increase in % $_{\mathrm{IMB}}$INH intensity was observed in D12-I DF compared to D12+I DF (15.74 vs 3.66±2.27, P<.01; Fig. 5-7 [left]).

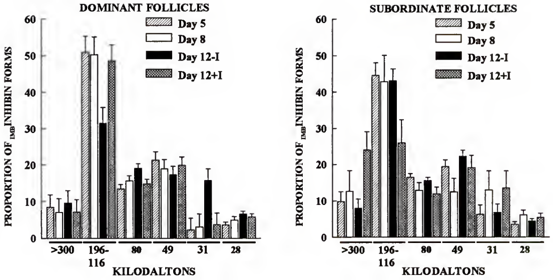

Figure 5-7. Least square means (±SEM) of proportion of $_{IMB}$INH intensity (α-subunits, α-β dimers; proportion of arbitrary densitometric units [AU] of each form [300, 196-116, 80, 49, 31 and 28 kDa] of inhibin/AU of total forms of inhibin) in first wave DF (left) and SF (right) of non-lactating dairy cows. Ten μg of total protein from each DF or SF were loaded per lane. Membranes were incubated with mink antiserum againt inhibin α_c^{1-26}gly.tyr, were washed and then were incubated with radiolabeled ^{125}I-inhibin α_c^{1-26}gly.tyr (^{125}I-INH).

The % $_{IMB}$INH was low in D5 and D8 DF. No effects of experimental group ($P<.20$; Fig. 5-7 [left and right]), follicle ($P<.20$; Fig. 5-7 [left and right]) or their interaction ($P<.54$; Fig. 5-7 [left and right]) were detected for the % of $_{IMB}$INH 28 kDa inhibin form.

Figure 5-8 (A) depicts the picture of a Northern blot of total RNA extracted from individual follicle walls (n=8, 2 follicles per experimental group) hybridized to α-inhibin cDNA. The picture of the ethidium bromide stained gel is depicted in Fig. 5-8 (B).

A

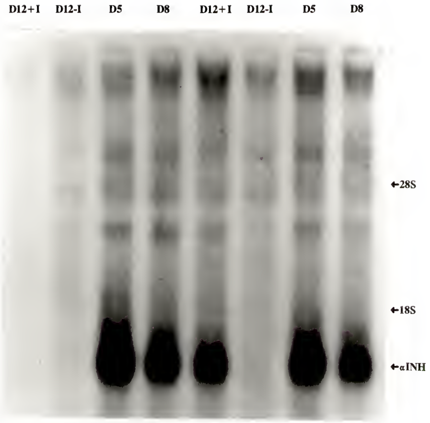

Figure 5-8 Northern blot analysis of α-inhibin. Ten μg of total RNA extracted from first wave DF at D5, D8, D12-I and D12+I were loaded per lane. A. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled α-inhibin cDNA. The hybridization signal was present at 1.3 Kb. B. Picture of ethidium bromide stained gel demostrating equal loading of RNA in each lane.

B

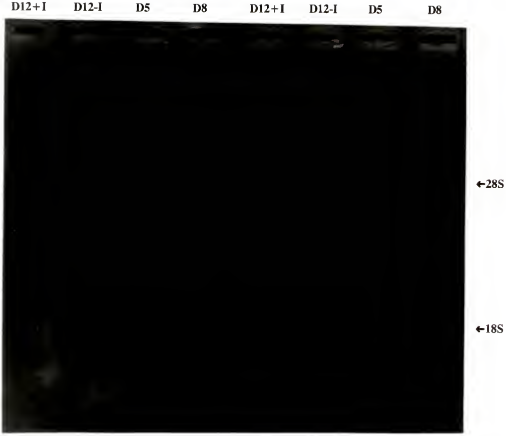

Figure 5-8. continued

The α-inhibin hybridization signal was present at 1.3 Kb. A hybridization signal for β_A-inhibin was not detected with preliminary Northern blot analyses, therefore, Poly $(A)^+$ RNA was used for a second set of Northern blot analyses. In Fig. 5-9 is depicted a Norther blot of Poly $(A)^+$ RNA extracted from a pool of D12+I DF walls (n=8) hybridized to β_A-inhibin cDNA. β_A-inhibin hybridization signal was present at 6.4 Kb.

Each individual follicle signal for both α-inhibin or β_A-inhibin mRNA in dot blots were adjusted for its corresponding 28S rRNA content to take into account potential differences due to unequal loading and transfer efficiency between samples. Pictures of representative sets of RNA dot blot analyses for α- and β_A-inhibin mRNA are shown in Fig. 5-10 and 5-11.

The mRNA levels for α-inhibin were higher in follicles of cows that were unilaterally ovariectomized (period 2) compared to those from intact cows (period 1; 252.51±17.16 vs 181.18±15.83, $P<.01$; Fig. 5-12, left). However, no interaction of period by experimental group ($P<.86$) or with follicle were detected ($P<.50$). Levels of α-inhibin mRNA had a different pattern of expression between experimental groups($P<.01$; Fig. 5-12, right) and between follicles (DF, 259.33±19.49 vs SF, 174.36±21.41, $P<.01$; Fig. 5-13, right). In D5 and D8 DF, expression of α-inhibin mRNA was higher compared to D12-I and D12+I DF (338.41±39.45 vs 175.60±41.47, $P<.01$; Fig. 5-12, right). In contrast, no differences were detected between D5 and D8 (347.41±35.68 vs 329.41±43.23, $P<.76$; Fig. 5-12, right) nor between D12-I and D12+I DF (181.95±47.01 vs 169.25±35.93, $P<.84$; Fig. 5-12, right). Moreover, no

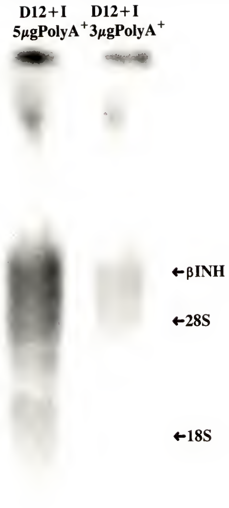

Figure 5-9 Northern blot analysis of β_A-inhibin. Five or three μg of Poly (A)$^+$ RNA from a pool of first wave D12+I DF were loaded per lane. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled β_A-inhibin cDNA. The hybridization signal was present at 6.4 Kb.

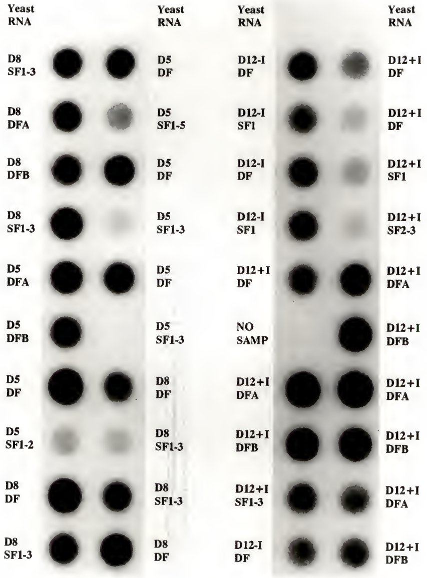

Figure 5-10. Dot blot analysis of α-inhibin. Five μg of total RNA extracted from first wave DF or SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled α-inhibin cDNA.

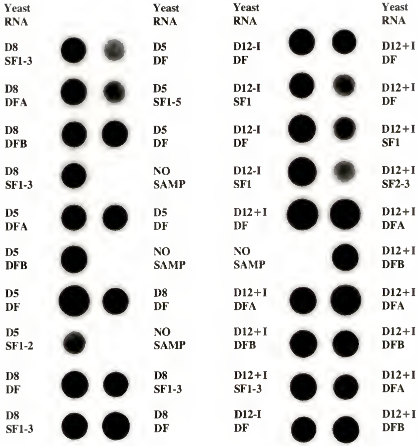

Figure 5-11. Dot blot analysis of β_A-inhibin. Five μg of total RNA extracted from first wave DF or SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled β_A-inhibin cDNA.

differences in levels of expression of α-inhibin were detected among experimental groups in SF ($P<.11$).

Analysis of follicular mRNA levels of β_A-inhibin in intact or in unilateral ovariectomized animals did not reveal differences in expression of message between follicles of either group (947.80±101.72 vs 899.59±95.89 vs $P<.76$). However, levels of β_A-inhibin mRNA had a different pattern of expression between experimental groups and between follicles (experimental group by follicle interaction, $P<.07$; Fig. 5-13). Dominant follicles at D5 and D8 had higher levels of message than did D12-I and D12+I DF (1482.63±214.82 vs 880.99±175.58, $P<.02$; Fig. 5-13). However, levels of mRNA did not differ between D5 and D8 DF (1433.03±188.18 vs 1532.23±241.47, $P<.70$; Fig. 5-13) nor between D12-I and D12+I DF (780.50±165.41 vs 981.48±185.75, $P<.42$; Fig. 5-13). A different trend was observed in SF. Here a difference in β_A-inhibin expression was only detected between D12-I and D12+I SF (813.32±99.41 vs 240.31±165.85, $P<.01$; Fig.5-13).

Correlation and Partial Correlation Studies

In Table 5-2 are depicted the simple (top-right) and partial correlations (bottom-left) coefficients between concentrations of $_{RIA}$INH (α-subunits, α-β dimers; ng/mg protein), amounts of 300, 196-116, 80, 49, 31 and 28 kDa inhibin forms ($_{IMB}$INH, α-subunits, α-β dimers; arbitrary units/10 μg total protein) and steady state mRNA levels (arbitrary units/5 μg of total RNA) of α and β_A-inhibin in DF. Most forms of $_{IMB}$INH (300, 196-116, 80, 49 and 28 kDa) were highly correlated with each other ($r>0.50$, $P<0.01$) but not with the 31 kDa form. In fact, the 31 kDa form was only correlated

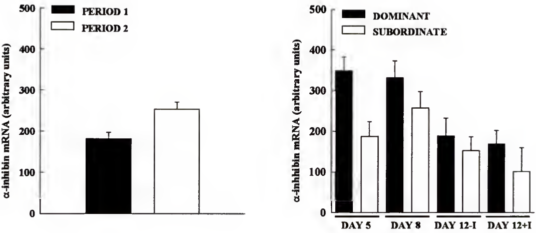

Figure 5-12. Least square means (±SEM) of hybridization intensity (arbitrary units) of α-inhibin cDNA to mRNA from first wave DF and SF of non-lactating dairy cows.

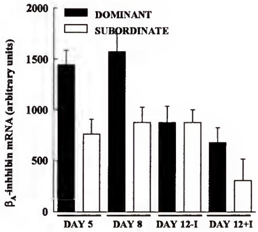

Figure 5-13. Least square means (±SEM) of hybridization intensity (arbitrary units) of β_A-inhibin cDNA to mRNA from first wave DF and SF of non-lactating dairy cows.

with the 28 kDa form ($r=0.49$, $P<0.01$). In general, $_{RIA}$INH was not associated with any form of $_{IMB}$INH ($r>0.07$, $P<0.11$). Levels of mRNA for α and β_A-inhibin were not correlated with each other nor with any form of $_{IMB}$INH.

Partial correlations between the same set of variables are depicted in Table 5-2 (bottom-left). These correlations have been adjusted for the effects of experimental group, period, experimental group by period and for cow variation. The 300, 196-116, 80, 49 and 28 kDa forms were not correlated with $_{RIA}$INH ($P>0.30$) concentrations whereas the 31 kDa form was associated with $_{RIA}$INH ($r=0.45$, $P=0.10$). Levels of mRNA for α and β_A-inhibin were correlated negatively with each other ($r=-0.55$, $P=0.04$) but not correlated with all the $_{IMB}$INH forms present in FF ($P<0.35$).

Simple and partial correlations were also calculated using $_{RIA}$INH contents per follicle (ng/follicle), contents of $_{IMB}$INH 300, 196-116, 80, 49, 31 and 28 kDa forms per follicle (arbitrary units/total protein/follicle) and mRNA contents of α and β_A-inhibin per follicle (arbitrary units/total RNA/follicle). Simple correlations are depicted in the top-right of Table 5-3 whereas partial correlations are depicted in the bottom-left of Table 5-3. The 300, 196-116, 80, 49, 31 and 28 kDa forms were highly correlated with $_{RIA}$INH ($r=0.30$ to 0.95, $P<0.05$), but were not correlated with levels of mRNA for α and β_A-inhibin ($r=0.04$ to 0.24, $P>0.08$). Levels of mRNA for α and β_A-inhibin were correlated between each other ($r=0.89$, $P<0.01$). The same trend was observed with the partial correlations (Table 5-3, bottom-left); however, the correlation coefficients and probability values tended to be higher with this statistical analysis compared to the former one.

Table 5-2. Simple (top right) and partial (bottom left) correlations between concentration of inhibin ($_{RIA}$INH; α-subunits, α- β dimers; ng/mg protein), amounts of 300, 196-116, 80, 49, 31 and 28 kDa inhibin forms ($_{IMB}$INH; α-subunits, α- β dimers; arbitrary units/10μg total protein) and steady state mRNA levels (arbitrary units/5μg total RNA) of α-inhibin and β_A-inhibin in dominant follicles. Within parenthesis are the P values for each coefficient.

▨	$_{RIA}$INH[a]	300[b]	196-116[c]	80[d]	49[e]	31[f]	28[g]	α-INH[h]	β_A-INH[i]
$_{RIA}$INH	▨	0.20 (0.19)	0.27 (0.07)	0.25 (0.09)	0.24 (0.11)	0.09 (0.56)	0.26 (0.08)	-0.08 (0.61)	0.10 (0.53)
300	0.20 (0.47)	▨	0.91 (<0.01)	0.88 (<0.01)	0.83 (<0.01)	0.16 (0.29)	0.52 (<0.01)	-0.16 (0.29)	-0.01 (0.92)
196-116	0.25 (0.37)	0.96 (<0.01)	▨	0.90 (<0.01)	0.85 (<0.01)	-0.01 (0.97)	0.55 (0.01)	-0.16 (0.32)	-0.05 (0.75)
80	0.30 (0.28)	0.91 (<0.01)	0.90 (<0.01)	▨	0.85 (<0.01)	0.25 (0.09)	0.64 (0.02)	-0.10 (0.51)	-0.07 (0.65)
49	0.08 (0.77)	0.72 (<0.01)	0.67 (<0.01)	0.81 (<0.01)	▨	0.21 (0.17)	0.75 (<0.01)	-0.07 (0.65)	-0.11 (0.48)
31	0.45 (0.10)	0.54 (0.04)	0.44 (0.11)	0.59 (0.02)	0.78 (<0.01)	▨	0.49 (<0.01)	0.12 (0.45)	-0.23 (0.13)
28	0.77 (0.79)	0.54 (0.04)	0.40 (0.14)	0.58 (0.02)	0.83 (<0.01)	0.82 (<0.01)	▨	-0.01 (0.96)	-0.17 (0.28)
α-INH	-0.13 (0.65)	0.02 (0.92)	0.05 (0.84)	0.19 (0.49)	0.22 (0.43)	0.25 (0.38)	0.27 (0.34)	▨	-0.22 (0.16)
β_A-INH	0.02 (0.93)	0.24 (0.40)	0.20 (0.47)	0.20 (0.48)	0.11 (0.69)	-0.08 (0.77)	-0.06 (0.81)	-0.55 (0.04)	▨

[a] $_{RIA}$INH,
[b] $_{IMB}$INH 300 kDa form, [c] $_{IMB}$INH 196-116 kDa form, [d] $_{IMB}$INH 80 kDa form, [e] $_{IMB}$INH 49 kDa form,
[f] $_{IMB}$INH 31 kDa form, [g] $_{IMB}$INH 28 kDa form,
[h] α-inhibin mRNA levels, [i] β_A-inhibinmRNA levels.

Table 5-3. Simple (top right) and partial (bottom left) correlations between contents of inhibin ($_{RIA}$INH; α-subunits, α- β dimers; ng/follicle), contents of 300, 196-116, 80, 49, 31 and 28 kDa inhibin forms ($_{IMB}$INH; α-subunits, α- β dimers; arbitrary units/follicle) and mRNA contents (arbitrary units/follicle wall) of α-inhibin and $β_A$-inhibin in dominant follicles. Within parenthesis are the P values for each coefficient.

	$_{RIA}$INH[a]	300[b]	196-116[c]	80[d]	49[e]	31[f]	28[g]	α-INH[h]	$β_A$-INH[i]
$_{RIA}$INH		0.68 (<0.01)	0.75 (<0.01)	0.75 (<0.01)	0.70 (<0.01)	0.32 (0.03)	0.59 (<0.01)	0.21 (0.19)	0.28 (0.07)
300	0.52 (0.05)		0.95 (<0.01)	0.94 (<0.01)	0.89 (<0.01)	0.40 (<0.01)	0.78 (<0.01)	0.04 (0.79)	0.08 (0.61)
196-116	0.53 (0.04)	0.97 (<0.01)		0.95 (<0.01)	0.91 (<0.01)	0.30 (0.04)	0.81 (<0.01)	0.09 (0.54)	0.12 (0.45)
80	0.66 (<0.01)	0.96 (<0.01)	0.96 (<0.01)		0.89 (<0.01)	0.43 (<0.01)	0.75 (<0.01)	0.08 (0.59)	0.09 (0.55)
49	0.34 (0.22)	0.85 (<0.01)	0.81 (<0.01)	0.81 (<0.01)		0.37 (0.01)	0.89 (<0.01)	0.20 (0.20)	0.18 (0.25)
31	0.59 (0.02)	0.79 (<0.01)	0.70 (<0.01)	0.79 (<0.01)	0.84 (<0.01)		0.55 (<0.01)	0.23 (0.14)	0.17 (0.26)
28	0.12 (0.66)	070 (<0.01)	0.65 (0.01)	0.60 (0.02)	0.93 (<0.01)	0.74 (<0.01)		0.27 (0.08)	0.24 (0.12)
α-INH	-0.24 (0.39)	-0.20 (0.49)	-0.25 (0.37)	-0.17 (0.55)	0.01 (0.95)	0.03 (0.91)	-0.04 (0.87)		0.89 (<0.01)
$β_A$-INH	-0.30 (0.28)	-0.32 (0.25)	-0.40 (0.15)	-0.31 (0.27)	-0.13 (0.64)	-0.11 (0.70)	-0.19 (0.49)	0.91 (<0.01)	

[a] $_{RIA}$INH,
[b] $_{IMB}$INH 300 kDa form, [c] $_{IMB}$INH 196-116 kDa form, [d] $_{IMB}$INH 80 kDa form, [e] $_{IMB}$INH 49 kDa form,
[f] $_{IMB}$INH 31 kDa form, [g] $_{IMB}$INH 28 kDa form),
[h] α-inhibin mRNA content, [i] $β_A$-inhibinmRNA content.

Stepwise Regression and Standardized Regression Coefficients

Standardized regression coefficients (SRC) calculated from stepwise regression analyses of FF contents of $_{RIA}$INH (α-subunits, α-β dimers; ng/follicle), contents of 300, 196-116, 80, 49, 31 and 28 kDa inhibin forms ($_{IMB}$INH, α-subunits, α-β dimers; arbitrary units/total protein/follicle) and mRNA contents (arbitrary units/total RNA/follicle) of α and β_A-inhibin in DF are depicted in Figure 5-14. Only SRC with probability values $\leq$ 0.15 were included in the diagram depicted in Fig. 5-14. Bolded filled arrows represent strong and positive associations whereas bolded dashed arrows represent negative associations. Thin arrows represent associations that were considered relatively less important or not important at all (based upon the relative magnitude of the SRC). The SRCs can be compared within variables but not among variables. For example, the association between the 80 kDa and the 31 kDa $_{IMB}$INH (1.72) can be compared to the association between the 300 kDa and the 31 kDa $_{IMB}$INH (0.65; Fig. 5-14). However, such a comparison cannot be made for the association between the 80 kDa and the 31 kDa $_{IMB}$INH (1.72) or the association for the 80 kDa and the 49 kDa $_{IMB}$INH (0.52; Fig. 5-14). A more thorough explanation of the methodology used to develop these SRC and how they are interpreted is presented in Chapter 4. Only two forms (49 and 28 kDa) of inhibin have been identified as monomeric forms in FF whereas five other forms ($>$160, 122-116-110, 80-77, 58 and 34) have been identified as being dimeric (Ireland and Ireland, 1994; Good *et al.,* 1994). Therefore, we followed this classification for analytical purposes (Fig. 5-14). The 196-116 kDa form had positive and strong associations with the 300 kDa form

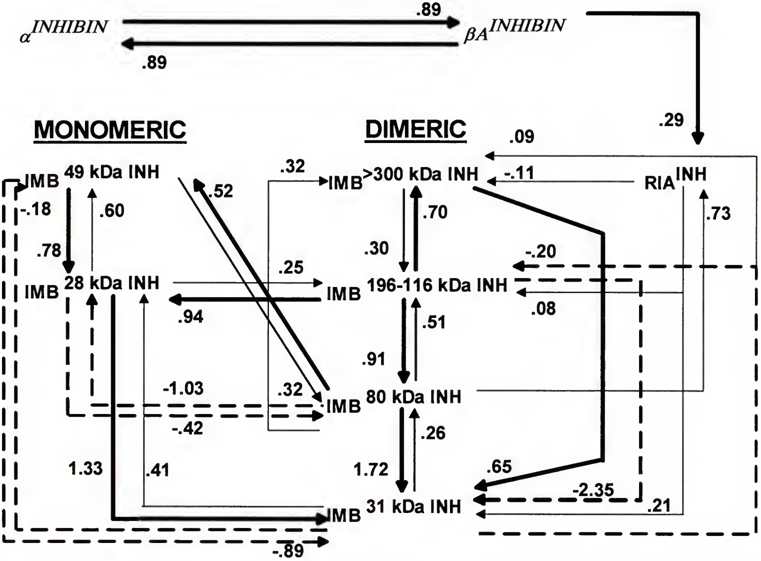

Figure 5-14. Standardized partial coefficients obtained from stepwise regression analysis of follicular fluid $_{RIA}$INH contents, $_{IMB}$INH forms (300, 196-116, 80, 49, 31 and 28 kDa) contents, and inhibin α and β_A-subunit mRNA contents in whole dominant follicles.

(0.70), the 80 kDa form (0.91) and with the 28 kDa form (0.94). Conversely, it had a strong negative association with the 31 kDa form (-2.35). The 80 kDa form had positive and strong associations with the 49 kDa (0.52) and 31 kDa (1.72) forms but had a negative association with the 28 kDa form (-1.03). The 31 kDa form was associated negatively with both the 196-116 kDa form (-2.35) and with the 49 kDa form (-0.89) but was positively associated with the 28 kDa form (1.33). Moreover, the 28 kDa form was negatively associated with the 80 kDa form (-0.42); the 49 kDa form was positively associated with the 28 kDa form (0.78). No associations were detected between any of $_{\mathrm{IMB}}$INH forms and mRNA levels of α and β_A-inhibin in DF. There was a slight degree of association between mRNA levels of β_A-inhibin and $_{\mathrm{RIA}}$INH (0.29).

Discussion

Changes in $_{\mathrm{RIA}}$INH concentrations, amounts of $_{\mathrm{IMB}}$INH forms and mRNA levels of α- and β_A-inhibin occurring in first wave dominant and subordinate follicles during four very distinct stages of follicular development were characterized in this study. In addition, these variables were studied by correlation, partial correlation and stepwise regression analyses to determine their potential involvement in the processes of dominance and atresia of dominant follicles.

Dominant and subordinate follicles were separated into two different populations based on the ratio of absolute concentrations of E_2 to P_4 or A_4 rather than on the basis of low or high level of these steroids (Ireland and Roche, 1982, 1983; Guilbault *et al.,* 1993). Therefore, EPR or EAR >1 were selected as thresholds to divide follicles (dominant and subordinates) into E_2-active (EPR or EAR > 1) or E_2-inactive (atretic, EPR or EAR ≤ 1; Ireland and Roche, 1982, 1983; Guilbault *et al.*, 1993). Based upon the EPR threshold (>1), all DF at D5 (12/12) and all but one DF at D8 (9/10) were E_2-active or non-atretic (Table 5-1). Conversely, all SF at D5 (12/12) and D8 (10/10) were E_2-inactive or atretic (Table 5-1). At D12-I, all but 2 DF (9/11) were atretic compared to D12+I in which only one DF was atretic and all other (10) DF were E_2-active (Table 5-1). The majority of SF at D12+I (9/11) were atretic whereas only 50% of SF were atretic at D12-I (Table 5-1). Similar conclusions can be obtained after using the EAR to separate follicles in E_2-active and E_2-inactive. However more SF at D12-I would fall in the E_2-active category using this ratio (Table 5-1). The presence of an atretic DF (D12-I) allowed follicle turnover and development of a new follicular wave; therefore, these newly recruited follicles are starting to develop A_4 and E_2 production capabilities. These observations would indicate that competence to produce A_4 is developed first compared to E_2, and that this is reflected by a higher frequency of SF with EAR>1 compared to EPR>1 (Table 5-1). Several studies have utilized the EPR and EAR alone (Ireland and Roche, 1982; Martin *et al.*, 1991) or with histological studies (i.e. E_2 active dominant follicle have <1% pycnotic cells, presence of mitotic figures, and integrity of at least 95% of granulosa cells against the basement membrane; Grimes and Ireland, 1986; Grimes *et al.*, 1987; Guilbault *et al.*, 1993; Ireland and Roche, 1983) to categorize ovarian follicles into dominant or E_2-active and atretic or E_2-inactive follicles. In the present study histological classification of follicles was not done because all tissue of the wall of each follicle was utilized for RNA extraction. However, the steroid profiles measured in individual follicles (Chapter 3 and 4) and the steroid ratios presented here follow the same pattern of those described previously by Ireland and Roche (1983) and by Guilbault *et al.* (1993). Thus, DF obtained from the D5 (E_2 active) and D8 (transitional E_2 active) experimental groups can be classified accurately as E_2-active follicles based upon the steroidal ratios in FF whereas all SF from these two experimental groups were accurately classified as E_2-inactive follicles. Conversely, the majority of DF at D12-I were classified as E_2-inactive (atretic, 9/11). In addition, the fate towards atresia that transitional DF at D8 normally follows during the first wave of follicle development was decelerated by injection of $PGF_{2\alpha}$ and insertion of a Norgestomet implant. Therefore, the majority of D12+I DF (10/11) remained estrogen active rather than becoming atretic after $PGF_{2\alpha}$ injection and insertion of a progestin implant at d8 of the estrous cycle.

The $_{RIA}$INH concentrations in FF remained fairly constant across all experimental groups studied (Fig. 5-2). However, DF had higher $_{RIA}$INH concentrations than SF. These results do not agree with those reported by Guilbault *et al.* (1993) and by Ireland and Ireland (1994) where RIA concentrations decreased with atresia of non-ovulatory follicles. To some extent, the differences in changes of $_{RIA}$INH concentrations between our study and other studies could reside on usage of different antibodies ([this study, mink-anti inhibin $\alpha_C^{1\text{-}26}$ gly.tyr]; [ewe s114/2-hInhibin $\alpha_C^{1\text{-}32}$, Ireland and Ireland, 1994; Guilbault *et al.*, 1993]) which ultimately would produce different affinities for the native inhibin molecules present in FF. Nevertheless, it is evident from these results and from those described elsewhere (Ireland and Ireland, 1994), that $_{RIA}$INH concentrations of inhibin (α-subunits and α-β dimers) do not reflect the dynamic changes of the different forms of Inhibin in FF that are detected by immunoblot analysis.

Qualitative immunoblot analysis of inhibin of non-reduced FF identified at least eight forms containing the $\alpha_C^{1\text{-}26}$ epitope (i.e. α-subunits or the α-β dimers; Fig. 5-3). The M_r of these forms (300, 196-116, 80, 58, 49, 31, 28 and 25 kDa) were in very close agreement with those reported by Ireland and Ireland (1994; >160, 122, 77, 58, 49, 48, 34 and 29 kDa) by immunoblot analysis using the same antibody. The 196-116 kDa forms detected in the present study appear to be composed of two different forms (196 and 116); although, the bulk of intensity was associated with the 116 kDa form, under the present assay conditions we were unable to obtain an individual densitometric reading for each form. More recently, Good *et al.* (1994), using an antibody against the β-subunit has further identified the 49 and 29 kDa bands as α-monomeric forms and the >160, 122-116-110, 80-77, 58, and 34 kDa bands as α-β dimeric forms in bovine FF. Therefore, it is very likely that the 49 and 28 kDa forms identified in this study are α-monomeric in nature and the remaining ones are α-β dimeric forms. This is the classification followed in Fig. 5-14. Approximately 25% of monomeric forms and of 75% of dimeric forms were present in all experimental groups and follicles (Fig. 5-7). Similar proportions could be calculated from the results reported by Ireland *et al.* (1994) based upon their immunoblot analysis. However, Knight *et al.* (1991) and Betteridge and Graven (1991) using IRMA reported that monomeric free α-subunits were present in excess compared to dimeric forms in FF. Schneyer *et al.* (1991) using a similar antibody (rabbit antiserum against human

Inhibin α_c^{1-25}gly.tyr) detected 57, 44, 42, 27 and 20 kDa forms thought to be products of postranslational processing of α-Inhibin in human FF, and similar immunoblot results have been reported by Miyamoto *et al.* (1986) and Sugino *et al.* (1992) for bovine FF.

Using an antibody specific to the α-inhibin subunit, Miyamoto *et al.* (1986) identified seven forms of inhibin (120, 108, 88 kDa [trimeric=dimeric +monomeric 62 kDa]; 65, 55, and 32 kDa [dimeric]; 62 kDa [monomeric]) in FF whereas Sugino *et al.* (1992) using the same antibody to the α-subunit and a separate one to β_A-subunit identified seven (105, 95, 55 and 32 kDa [dimeric]; 50 and 40 kDa [αmonomeric]; 55 kDa [$\beta_A$monomeric]) forms of inhibin in FF. Although large $_{IMB}$INH (116-122 kDa) forms found in D5 and D8 DF are likely to represent large precursors of dimers secreted into FF (Sugino *et al.*, 1992), super-large dimeric forms (196 and >300 kDa) described by us and others (Ireland *et al.*, 1994) could represent the formation of large non-specific aggregates between the 116-122 kDa forms and free α-subunits of inhibin, between free α-subunits or between dimeric forms of inhibin and follistatin. Indeed, inhibin has one binding site and activin has two binding sites for follistatin, and Shimonaka *et al.* (1991) hypothesized that follistatin binds activin and inhibin through their common β-subunits. Regardless of the nature of association between the $_{IMB}$INH forms present in FF or between these forms and follistatin, these associations are somewhat resistant to the denaturing conditions of SDS-PAGE, since large M_r forms remain intact after the immunoblot analysis. Moreover, isolation, purification, sequencing and determination of biophysical characteristics of each $_{IMB}$INH form remains to be elucidated before defined roles can be attributed to each of them. The results reported in this study as well as those by Ireland *et al.* (1994) lend further support to the theory that in bovine FF, inhibin is secreted as a large precursor protein by granulosa cells and then processed through proteolytic cleavage (postranslational modifications) into smaller M_r monomeric and dimeric forms (Sugino *et al.*, 1992).

Two very distinct patterns of change for the amounts of $_{IMB}$INH forms were observed in FF of DF and SF across the four experimental groups studied. Absolute amounts of four $_{IMB}$INH forms (>300, 196-116, 80 and 49 kDa) were high in E_2 active (D5) and E_2 active transitional (D8) DF (EPR>1), but were decreased with atresia (D12-I) DF (EPR≤1; Fig. 5-5, all four panels). However, absolute amounts of these $_{IMB}$INH forms tended to remain at higher levels (albeit sometimes not significant) in E_2 active-sustained DF (D12+I; EPR>1) compared to those present in atretic follicles (Fig. 5-5, all four panels). Conversely, absolute amounts of the 31 kDa form of inhibin in FF steadily increased with atresia of the DF (D5<D8<D12-I). At d12, atretic follicles (D12-I; EPR≤1) had twice the amounts of the 31 kDa form compared to E_2 active-sustained DF (D12+I; EPR>1; Fig. 5-6, left). Although the presence of different M_r forms of Inhibin in bovine FF has been described previously by Miyamoto *et al.* (1986), Schneyer *et al.* (1991) and Sugino *et al.*, (1992), the absolute changes in amounts of inhibin forms in FF has been described only recently by Ireland *et al.* (1994) using ovarian follicles at various physiological stages that were obtained from a local abattoir.

In the present study, dominant and subordinate follicles at four defined stages of development (E_2 active [D5; EPR>1], E_2 active transitional [D8; EPR>1], atretic [D12-

I; EPR≤1] and E_2 active-sustained [D12+I; EPR>1]) were collected at ovariectomy and used to characterize changes in different forms of inhibin by immunoblot analysis and in an experimental group in which the function of the first wave DF is sustained. Therefore, the utilization of this experimental model has enabled us to study temporal changes in absolute amounts of inhibins across the first wave of follicle development. The results reported here suggest that as the dominant follicle undergoes progressive changes from E_2 active [D5; EPR>1], E_2 active transitional [D8; EPR>1] and atretic [D12-I; EPR≤1], the total $_{IMB}$INH amounts (Fig. 5-4) and at least four forms of inhibin (300, 196-116, 80 and 49 kDa) present in FF decrease 50 to 75% (Fig. 5-5). These temporal changes follow a decline in absolute E_2 and A_4 concentrations in FF (Chapter 4, Fig. 4-2; Chapter 3, Fig. 3-7) as well as a decrease in the EPR and EAR from >1 to ≤1 in which the latter follicles (D12-I) are functionally atretic (Fig 5-1; Table 5-1; Ireland and Roche, 1982, 1983; Grimes *et al.,* 1987; Guilbault *et al.,* 1993; Martin *et al.,* 1991). Furthermore, an opposite pattern was observed with the 31 kDa form. In this case, an increase of 50% in absolute amounts of $_{IMB}$INH 31 kDa form (Fig. 5-6, left) was associated with an increase in absolute P_4 concentrations in FF (Chapter 3, Fig. 3-7) and with the development of functional atresia (EPR, EAR ≤1, D12-I; Table 5-1) of the D12-I DF. Subordinate follicles across all experimental groups studied had the same pattern of change in absolute amounts of total $_{IMB}$INH, of $_{IMB}$INH forms (300, 196-116, 80, 49 and 31 kDa; Fig 5-4; 5-5 ; 5-6, left) and of the steroids profiles (Fig. 5-1 and Table 5-1) in FF compared to those reported for D12-I atretic DF. In SF, total $_{IMB}$INH and amounts of the $_{IMB}$INH 300, 196-116, 80 and 49 kDa forms were 25 to 70% lower than those present in a D5 E_2 active DF. Conversely, the amounts of 31 kDa $_{IMB}$INH form were 30 to 45% increased in FF of SF (Fig. 5-6, left). Ireland *et al.* (1994) recently reported absolute changes in different forms of inhibin present in FF of E_2 active (P_4:E_2 ratio <1 [PER]), atretic (PER=1 to100) and highly atretic (PER= > 100) follicles from ovaries obtained from the abattoir. In agreement with the present study, they reported decreases in absolute amounts of total $_{IMB}$INH and in the >160, 122, 77, 68, 58, 49 and 48 kDa forms and an increase in absolute amounts of the 31 kDa form in FF with highly atretic follicles.

The proportion of different $_{IMB}$INH forms relative to the total amounts present in FF changed during follicular development in first wave dominant and subordinate follicles (Fig. 5-7, left and right). In E_2 active (D5; EPR>1), E_2 active transitional (D8; EPR>1) and E_2 active-sustained (D12+I; EPR>1) DF, the predominant form of inhibin was the 196-116 kDa form which represented approximately 50% of the total forms present in FF (Fig. 5-7, left). However, in atretic (D12-I; EPR≤1) DF the 196-116 kDa forms were reduced to only 30% of total forms present in FF. In addition to this reduction in the proportion of the 196-116 kDa form was an increase in the proportion of the 31 kDa form. In D5, D8 and D12+I DF the proportion of the 31 kDa form was 3-5% and this increased to 15% in atretic DF (D12-I).

The proportion of the 300 and the 80 kDa forms also increased slightly with atresia in the DF (D12-I; Fig. 5-7, left). In SF the proportion of the 196-116 kDa form represented 40% of total $_{IMB}$INH forms in FF compared to DF where it represented 50%

(Fig. 5-7, right). This 10 % decrease in the proportion of the 196-116 kDa form was associated with an increase in all other forms present in the FF. The compensatory increase was evident in the >300 and 31 kDa forms. In D12+I SF, a significant reduction in the 196-116 forms was associated with an increase in the 300 and 31 kDa forms whereas in D12-I SF the 300 and 31 kDa forms remained low and the 196-116 remained high (Fig. 5-7, right). Therefore, under the influence of a strong E_2 active sustained D12+I DF, 82% of the SF in this experimental group were atretic (EPR≤1) and had increased amounts of the 300 and the 31 kDa forms. Conversely, in the presence of an atretic D12-I DF, follicle turnover was taking place and a "second wave" of follicle recruitment and selection was underway as reflected by the fact that only 54% of the D12-I SF were atretic (EPR≤1; Table 5-1), and increased amounts of the 196-116 kDa and decreased amounts of 300 and 31 forms of inhibin were present. In the study of Ireland *et al.* (1994), the proportion of the 122, 49 and 48 kDa forms decreased, the proportion of the 29 kDa form remained unchanged and the proportion of the 31 kDa form increased in association with atresia. In our study, similar results were obtained, but we report changes in the proportions of $_{IMB}$INH between dominant and subordinate follicles at precise stages of follicle development and during programmed alterations in follicle development. Using our experimental model, the functional status of each follicle was predicted according to the precise day of the estrous cycle the follicle was collected at ovariectomy, and this was further confirmed by the ratio of steroidal concentrations in FF. Thus, either absolute values or the proportion of each $_{IMB}$INH form present in FF could be related to the status of the follicle (dominant, atretic, subordinate) and to the "day of the wave". Collectively, these results as well as those reported elsewhere (Ireland et al., 1994) indicate that the absolute amounts and proportion of $_{IMB}$INH forms present in follicular fluid undergo very dynamic qualitative and quantitative changes during the periods of follicular dominance and atresia of non-ovulatory follicles that are not accurately measured by $_{RIA}$INH. Moreover, these changes reflect a potentially complex mechanism for postranslational processing of inhibin forms that yet remains to be elucidated.

We chose to isolate total RNA from whole follicle walls because it has been documented extensively that granulosa cells of follicles are the primary source of Inhibins in cattle (Rodgers *et al.,* 1989; Torney *et al.,* 1989). This allowed us to obtain sufficient amounts of total RNA to be able to probe for different mRNAs within individual DF and SF. Northern blot analysis of individual dominant and subordinate follicles detected a single hybridization signal at 1.3 Kb for α-inhibin (Fig.5-8) in agreement with the size of the original bovine cDNAs cloned (1.1-1.3 Kb; Forage *et al.,* 1986). Moreover, using pools of selected bovine follicles (i.e., pooled by size and steroidal concentrations) for Northern blot analysis, Juengle *et al.* (1993) found a single hybridization band (1.8 Kb, rat cDNA probe) whereas Ireland and Ireland (1994) and Rodgers *et al.* (1989) found multiple mRNAs for α-inhibin. A predominant band at 1.3 Kb and additional bands at 2.2, 3.3 and 6.4 Kb were detected using a human cDNA probe (Ireland and Ireland, 1994) whereas a strong hybridization signal at 1.7 Kb and a much weaker one at 3.0 Kb was detected with a bovine probe in the second study (Rodgers *et al.,* 1989). A single message for α-inhibin was detected by Northern blot analysis of individual samples with an approximate size of 1.3 Kb in the present study. Northern blots to identify β_A-inhibin message were performed initially on total RNA isolated from individual follicles. However, this system was unsuccessful in identifying β_A-inhibin message even after a 15-fold increase in exposure time for autoradiography. When Northern blot analysis was done using poly(A)$^+$ RNA from a pool of dominant follicles, a single hybridization signal at 6.4 Kb for β_A-inhibin was detected (Fig.5-10). Previous studies using a bovine cDNA probe (Rodgers *et al.,* 1989) detected a single hybridization band at 6.5 Kb and a less predominant band at 6.9 Kb whereas studies using a human cDNA probe detected 6.4 Kb band and a less predominant 2.6 and 4.0 Kb bands (Ireland and Ireland, 1994) and 5.7 and a minor band at 4.7 Kb (Juengel *et al.,* 1993). Furthermore, Rodgers *et al.,* (1989) reported the same technical difficulties in detecting β_A-inhibin message in cattle ovarian tissue and speculated that α-inhibin mRNA levels were much greater than β_A-inhibin mRNA levels in bovine follicles. Similar and confirmatory findings have been reported for ovaries of the pig (Mason *et al.,* 1985) and rats (Esch *et al.,* 1987; Meunier *et al.,* 1988) suggesting that β_A-inhibin genes are either expressed at a much lower rate or that β_A-inhibin mRNA has a much shorter half-life (Rodgers *et al.,* 1989).

The α-inhibin mRNA levels were increased in follicles from unilateral ovariectomized cows compared to intact cows. We previously reported that the size of the DF and dominance (difference in size between DF and SF) were larger, and that mRNA expression of P450 SCC and P450 3β-HSD were elevated in unilateral ovariectomized cows (Chapter 3). In addition, we also reported that mRNA expression of P450 17α-HYD and P450 ARO were also elevated in follicles obtained from ovariectomized cows (Chapter 5). However, no interactions of experimental group by period or experimental group by follicle was detected. Therefore, removal of one ovary during the first period, induced a major alteration in ovarian feedback, presumably to enhance gonadotrophin secretion, and this strong gonadotropin feed-back from the pituitary may have induced recruitment and selection of a DF earlier in the first wave (Chapter 3; Fig. 3-2, left and right). This effect also altered gene expression for the steroidogenic enzymes and α-inhibin in association with a greater dominance of the DF. Further support for greater follicular dominance in unilateral ovariectomized cows was that the number of class 1 follicles (3-5 mm) was decreased during the same period of time (Chapter 3; Fig. 3-3).

Levels of α-inhibin and β_A-inhibin mRNA were elevated during the first 8 d of the estrous cycle but then decreased with atresia of the DF (D12-I; Fig 5-12, right; Fig.5-13); injection of $PGF_{2\alpha}$ and the insertion of a progestin implant at d8 did not modify this trend in D12+I DF (D12-I; Fig 5-12, right; Fig.5-13). In addition, mRNA levels for α- β_A-inhibin decreased by 40% in SF across all experimental groups compared to DF (Fig. 5-12, right; 5-13). However, D12-I SF had higher mRNA levels for β_A-inhibin compared to D12+I SF (Fig 5-13). In the former group, because the presence of an atretic DF, follicle turnover and a new wave of follicle recruitment is underway; however in the latter group, the presence of an E_2 active sustained DF inhibits new follicle recruitment. Similar, albeit non-significant differences, were observed with mRNA levels for α-inhibin (Fig. 5-12, right). Rodgers *et al.* (1989) detected mRNA for both inhibin subunits (i.e. α, β_A) in pools of bovine follicles (< 3mm, 4-6 mm, 7-10 mm); however, no indication of the steroid concentrations in FF nor the stage of development of the follicles (dominant, atretic, subordinate, etc.) was reported. Nevertheless, mRNA levels for α-subunit were detectable in all three follicle pools studied whereas mRNA levels for β_A-subunit was only detectable in the 7-10 mm pool of follicles when a cDNA probe was used. However, β_A-subunit mRNAs were detectable in all three pools of follicles when a cRNA probe was used. In a different study by Ireland and Ireland (1994), mRNA levels for inhibin α- and β_A-subunits decreased with atresia. Ovarian follicles were obtained from the slaughterhouse and classified as either E_2 active (EPR > 1), atretic (P_4:E_2 ratio [PER] 1 to 100) or highly atretic (PERT > 100) based upon steroid ratios in FF. Messenger RNA levels for inhibin α- and β_A-subunits were 40% lower in atretic follicles and 75% lower in highly atretic follicles compared to E_2 active follicles. Collectively, results of the present study indicate that levels of mRNA for inhibin α- and β_A-subunits decrease with atresia of the DF and the increase in the absolute amounts and proportions of $_{\text{IMB}}$INH observed in D12+I DF (E_2 active sustained) are more likely due to processing of preexisting forms rather than *de novo* synthesis of these forms. Associated with these differences in recruitment among SF was an increase in gene expressionin E_2 active SF.

The secondary FSH surge occurring on days -1 and 0 of the estrous cycle (Adams *et al.,* 1992) recruits a cohort of ovarian follicles into the potential ovulatory pool and is responsible for the initiation of expression for inhibin subunit mRNA levels during early stages of folliculogenesis (D'Agostino *et al.,*1991). As a consequence of this FSH stimulation, inhibin produced by granulosa cells feeds-back through an endocrine loop to reduce pituitary FSH production (Findlay, 1993; Hasegawa *et al.,* 1987; Hillier, 1994).

Further evidence for this endocrine feed-back is provided by the subsequent increase in basal FSH concentrations, decreases in circulating inhibin concentrations, and the increased ovulation rate observed in heifers following immunization against α_N portion of the 58 kDa inhibin (Findlay *et al.*,1989; Russell *et al.*, 1994). In addition, Badinga *et al.* (1992) described an increase in plasma FSH and in the number of class 1 (3-4 mm) ovarian follicles in cows that were ovariectomized at d5 and d8 of the estrous cycle (DF was present) but no increase in plasma FSH was observed in cows with an atretic DF at d12. Although eight different forms were detected by immunoblot analysis ($>$300, 196-116, 80, 58, 49, 31, 28 and 25; Fig. 5-3) in bovine FF, two forms (58 and 25 kDa) were detected in only 11 and 7 of the 105 individual follicles studied and can be potentially disregarded from exerting negative feed-back at the pituitary level. Two other forms are α-monomeric in nature (49 and 28 kDa; Good *et al.*, 1994) and thus the same rationale can be followed because these forms do not exert endocrine feed-back at the level of pituitary (Knight *et al.*, 1989; Robertson *et al.*, 1989). Furthermore, the proportion of the 31 and the 80 kDa forms are lower in E_2-active (D5 and D8) compared to atretic (D12-I; Fig. 5-7) DF and thus are unlikely candidates for regulating pituitary FSH secretion at this early stage of the estrous cycle (d 5 and 8). Therefore the most likely candidate for exerting negative feedback at the pituitary level is the 196-116 kDa form because it is the most abundant form in E_2-active (D5 and D8) DF and because it exhibits a dramatic drop in atretic follicles (D12-I, Fig. 5-7). Further evidence in support of this idea is provided by the observation that levels of the 196-116 kDa form are maintained in E_2 active-sustained DF (Fig. 5-7) in which turnover of the first wave DF does not take place

(Chapter 3, Fig. 3-4) and follicular dominance is sustained because the majority of SF are atretic (9/11, Table 5-1) in this experimental group. Nevertheless, it is noteworthy to point out that although there is an intrafollicular pool of $_{\mathrm{IMB}}$INH, the isolation, sequencing and determination of biological activities of each form with specific bioassays remains to be elucidated.

Besides the classical endocrine loop described above, there is strong evidence supporting additional paracrine and autocrine regulatory loops for inhibin-like molecules within the ovary (Hillier *et al.*, 1991a, 1991b; Hsueh *et al.*, 1987; Schneyer *et al.*, 1991; Woodruff *et al.*, 1990). Inhibin produced by granulosa cells exerts a potent and selective stimulation of androgen synthesis in cultured theca cells (Hillier *et al.*, 1991a, 1991b; Hsueh *et al.*, 1987) and participates in local regulation of folliculogenesis (Woodruff *et al.*, 1990). This preferential stimulation of androgen production relative to progesterone production points to an action of inhibin on P450 17α-HYD, the rate limiting enzyme for thecal androgen biosynthesis (Hsueh *et al.*, 1987). Furthermore, this stimulation is more pronounced in the presence of LH and IGF-I (Hillier *et al.*, 1991a, 1991b). However, the results reported in this study do not support a strong role of inhibins in stimulating production of androgens. A sharp drop in A_4 concentrations already was detected in D8 DF (Chapter 4; Fig. 4-2, left). However, a 50% reduction in total absolute $_{\mathrm{IMB}}$INH and a 20% reduction in the 196-116 form of inhibin were only observed by d12 in D12-I atretic follicles (Fig. 5-4 and 5-5). In addition, P450 17α-HYD mRNA levels (Chapter 4, Fig. 4-6) did not decrease at the same time that FF A_4 concentrations did suggesting that P450 17α-HYD enzyme activity and not mRNA abundance was the rate limiting step. This reinforces the view that inhibin may not be playing an important role in regulating androgen production.

Regarding the roles of LH and IGF-I (Hillier *et al.*, 1991a, 1991b) possibly enhancing the autocrine and paracrine actions of inhibin at the follicular level, the data presented in Chapter 6 (Fig. 6-6) show that IGF-I concentrations of DF do not change among experimental groups. Moreover, the presence of low molecular weight IGFBPs -2, -4 and -5 in atretic D12-I DF was very likely binding IGF-I that were present in FF and therefore reducing the trophic activity by IGF-I in D12-I DF (atretic) compared to D5 and D8 DF (Chapter 6, Fig. 6-9 and 6-10). This increase in IGFBPs (Chapter 6, Fig. 6-9 and 6-10) coincides with a decrease in A_4 and E_2 concentrations in FF (Chapter 4, Fig. 4-2), with a decrease in total absolute $_{IMB}$INH in FF (Fig. 5-4) and with an increase in the 31 kDa form of $_{IMB}$INH (Fig. 5-6, left); therefore, collectively these observations provide additional support to the idea that IGF-I may have synergistic effects with LH over regulating inhibin production.

Additional paracrine and autocrine effects of α-subunit inhibin precursors through modulation of FSH receptor binding and bioactivity have been described by Sato *et al.* (1982) and Schneyer *et al.* (1991). In the present study, we did not distinguish monomeric from dimeric $_{IMB}$INH forms. However, based upon results from Good *et al.* (1994), the 49 and the 28 kDa forms identified in this study are most likely to be monomeric α-subunits. Therefore, these forms may be involved in the autocrine and paracrine mechanisms described above. Indeed, the 49 kDa form is high in E_2 active and transitional E_2 active DF (Fig. 5-5, bottom-right) possibly being secreted and taken up from the periphery by SF of both ovaries and binding to FSH receptors which would block recruitment of SF.

Levels of mRNA for α- and β_A-subunits (Fig. 5-12, left; Fig. 5-13) and absolute amounts of total and four out of six $_{IMB}$INH forms (Fig. 5-4; Fig. 5-5, >300, 196-116, 80 and 49 kDa) reach the highest levels around d 5 of the estrous cycle and remain at similar levels until d8. After d8, mRNA levels for α- and β_A-subunits and absolute amounts of $_{IMB}$INH (>300, 196-116, 80 and 49) decrease 35 to 70%. The injection of $PGF_{2\alpha}$ and the insertion of a progestin implant at d 8 of the estrous cycle in the D12+I experimental group maintained absolute amounts of these $_{IMB}$INH forms to levels similar albeit sometimes lower than those observed at D5 and D8 (Fig. 5-4; Fig. 5-5; Fig. 5-7). However, at D12+I, mRNA levels for both inhibin subunits remained at similarly low levels to those present in D12-I atretic DF. For example the proportion of the 196-116 kDa form decreased with atresia (D12-I) but remained high in E_2 active-sustained DF (D12+I) and just the opposite occurred with the 80 kDa and the 31 kDa forms; however no changes in the mRNA for α- and β_A-subunits were detected in both groups (Fig. 5-7; Fig. 5-12, left; fig. 5-13). Therefore, these changes represent striking examples of intrafollicular processing of inhibin molecules from higher to lower molecular weight forms during atresia of the DF.

Additional insights into this process were obtained by correlation, partial correlation and stepwise regression analyses (Table 5-2; Table 5-3; Fig. 5-13). These analyses confirmed our observations that absolute amounts of $_{IMB}$INH forms in FF were not correlated with mRNA levels for α- and β_A-subunits (Table 5-2; Table 5-3), and that $_{RIA}$INH concentrations were not correlated with absolute amounts of $_{IMB}$INH forms in FF (Table 5-2). This lack of correlation between $_{RIA}$INH concentrations and absolute contents of $_{IMB}$INH forms in FF is very likely to represent lack of sensitivity of the RIA to detect quantitative and qualitative changes in $_{IMB}$INH forms. The only differences detected by RIA were differences between dominant and subordinate follicles which were also observed with $_{IMB}$INH. However, changes in the proportion of the different forms of inhibin between experimental groups were not detected by $_{RIA}$INH. The observation that mRNA levels for α- β_A-inhibin were not correlated with the absolute amounts of $_{IMB}$INH in FF gives further support to the idea that intrafollicular processing of larger M_r to smaller forms rather than *de novo* synthesis is the mechanism for producing $_{IMB}$INH forms involved in the process of atresia of DF (D12-I). The use of stepwise regression analysis helped to gain additional information about potential processing routes among the inhibin molecules throughout the stages of DF function (Fig. 5-14). The observations that the 196-116 kDa form of inhibin is the most abundant form present in FF, that they decrease with atresia, that the majority of the intensity was present in the 116 kDa M_r form (Fig. 5-3), and that very strong positive associations exist with other forms present in FF suggest that this form is the one secreted into the FF by granulosa cells (116 kDa, Knight *et al.*, 1991; 120 kDa, Miyamoto *et al.*, 1986; 105 kDa, Sugino *et al.*, 1989). Two potential processing routes were detected (Fig. 5-14). The first processing route included the stepwise reductions from 196-116 to 80 kDa (0.91) and from 80 to 31 kDa (1.72). The strong negative association (-2.35) between 196-116 and 31 kDa confirmed this step indicating that when the amounts of 196-116 decreased the amounts of the 31 kDa form increased (Fig. 5-7). This potential processing route involves an intermediate step from 196-116 to 80 kDa form (0.91) and takes place during atresia of the DF (D12-I) because the proportion of the 80 kDa form is increased with atresia (Fig. 5-7). The second processing route involves a single step from 196-116 to the 300 kDa form (0.70), and this route is likely to be associated with the process of atresia of the DF because the proportion of this form increased in D12-I DF (Fig. 5-7). It is likely that this large molecular weight form represents an aggregation of inhibin molecules. Two additional processing routes may be operational involving protease breakdown of the 80 kDa form to the 49 kDa (0.52) form and subsequent enzymatic degradation to the 28 kDa (0.78) form. This route of processing would produce free monomeric subunits representing 25% of total $_{\mathrm{IMB}}$INH forms in FF. Further support for the processing of inhibin through this route is the negative association (-1.03) between the 80 and the 28 kDa forms (i.e. a unit change in the 80 kDa form is associated with a -1.03 unit decrease in the 28 kDa form in FF). The other processing route originates with the 196-116 kDa dimeric form being degraded to the 28 kDa monomeric form (0.94). However caution should be taken regarding the interpretation of these two additional routes since contradiction between the stepwise analysis and the observed results exist (i.e. only the 31 kDa form and not the 28 and 31 kDa forms increase with atresia).

In summary, the following conclusions can be drawn from the present study: 1) The majority of D5, D8 and D12+I DF were E_2 active (EPR > 1) whereas the majority of D12-I DF and SF were E_2 inactive (EPR≤1), 2) $_{RIA}$INH of FF does not reflect dynamic changes of the diverse FF inhibins detected by $_{IMB}$INH, 2) the proportions of the >300, 80 and 31 kDa $_{IMB}$INH forms increase during the process of atresia of the DF, 3) the 196-116 kDa $_{IMB}$INH form decreases during the process of atresia of the DF, 4) levels of mRNA for α- and β_A-subunits decrease with atresia of the DF and are not maintained in E_2 active-sustained DF (D12+I), 5) the dramatic changes in FF $_{IMB}$INH did not parallel the changes in mRNA levels for α- and β_A-subunits, suggesting that postranslational modifications (processing) of high molecular weight forms of $_{IMB}$INH are very likely the major mechanism that regulates inhibin levels if FF during atresia and sustained dominance of dominant follicles, 6) utilizing the technique of SPC, potential intrafollicular processing pathways were identified, and 7) potential forms of FF $_{IMB}$INH have been identified as likely candidates to potentially regulate FSH secretion (196-116 kDa) and to potentially exert an intraovarian regulatory effect to block recruitment (49 kDa).

CHAPTER 6

INSULIN-LIKE GROWTH FACTOR-I (IGF), IGF-II AND IGF-BINDING PROTEINS (IGFBP) CONCENTRATIONS IN FOLLICULAR FLUID AND IGFBP-2 mRNA LEVELS IN BOVINE FIRST WAVE DOMINANT AND SUBORDINATE FOLLICLE

Introduction

Folliculogenesis in mammalian species is a highly selective process. Only a very small proportion of total follicles (≈0.1%) survive atresia and give rise to dominant follicles (Aria, 1920). Probably the single most important question in folliculogenesis concerns the mechanisms by which a single follicle within a cohort is selected to become dominant or undergo programmed cell death by atresia (Erickson *et al.,* 1994). Follicular development is thought to be regulated primarily by the gonadotropic hormones, FSH and LH (Ireland and Roche, 1987; Lucy *et al.,* 1992; Richards, 1980). Evidence is also emerging that growth factors which are themselves the products of gonadotropin action, may act locally on follicle cells to either amplify or attenuate the timing and direction of differentiation (Erickson *et al.,* 1994; Hillier, 1994). Insulin-like growth factor-I is produced by granulosa cells the under control of FSH and E_2 (Hammond *et al.,* 1985) and has a synergistic effect with FSH to stimulate granulosa cells *in vitro* (Adashi *et al.,* 1991; Giudice, 1992; Hammond *et al.,* 1991). Expression of the IGF-I gene has been identified in granulosa cells of rats (Hernandez *et al.,* 1989; Oliver *et al.,* 1989) and cattle

(Echternkamp *et al.,* 1991; Spicer *et al.,* 1993). Receptors for IGF-I have been localized to rat (Adashi *et al.,* 1988; Davoren *et al.,* 1986) and porcine (Barano and Hammond, 1984) granulosa cells and rat theca cells (Hernandez *et al.,*1991). Conversely, IGF-II gene expression has been identified in rat theca cells (Hernandez *et al.,* 1990). In vitro studies have shown that IGF-I stimulates aromatase activity in rat granulosa cells (Adashi *et al.,* 1985a,b), human (Bergh *et al.,*1991; Erickson *et al.,*1989,1990; Christman *et al.,* 1991) and porcine granulosa cells (Veldhuis *et al.,*1985,1986,1987); P450 aromatase mRNA expression in human granulosa cells (Steinkamph *et al.,* 1988), P450 side chain cleavage enzyme mRNA expression in rat theca (Magoffin *et al.,* 1990) and porcine granulosa cells (Urban *et al.,*1990); LH receptor synthesis in rat granulosa cells (Adashi *et al.,*1985c); inhibin secretion in rat granulosa cells (Bicsak *et al.,* 1986) and androgen biosynthesis in rat theca cells (Hernandez *et al.,* 1988). Although changes in IGF-I and IGF-II concentrations have been reported for bovine follicular fluid (FF) and plasma (Badinga *et al.*, 1992; Echternkamp *et al.*, 1994; Spicer and Enright, 1991; Stanko *et al.,*1994), the relative contributions of IGF-I measured in bovine FF due to absorption from serum vs local secretion by granulosa cells of ovarian follicles are unknown (Echternkamp *et al.,*1990). Whereas Badinga *et al.* (1992) reported no changes in follicular fluid (FF) concentrations of IGF-I in first wave dominant follicles collected on days (d) 5, 8 and 12 of the estrous cycle, Echternkamp *et al.* (1994) reported that FF IGF-I concentrations increased with increasing concentrations of E_2 in FF.

An increasing body of evidence suggests that in the ovary, biological responses of IGF-I and IGF-II under normal FSH stimulation may also be regulated by the IGFBPs (Ui *et al.,* 1989). The IGFBPs may bind to IGF-I and IGF-II present in the FF and therefore selectively negate their trophic activity (Ui *et al.,* 1989; Magoffin and Erickson, 1994). Using ligand blot analysis, immunoprecipitation, in situ hybridization and Northern blot analysis, six IGFBPs (hIGFBP-1 [28 kDa], -2 [34 kDa], -3 [43&40 kDa], -4 [30&24 kDa], -5 31-29 kDa], and -6 [22 kDa]) have been identified in the human ovary (Giudice, 1992; Zhou and Bondy, 1994), five IGFBPs (rIGFBP-2 [30.5 kDa], -3 45&40 kDa], -4 [28&24 kDa], -5 [30&29 kDa], and -6 [26.5 kDa]) in the rat ovary (Giudice, 1992; Liu *et al.,* 1993; Nakatani *et al.,* 1991) and (pIGFBP-2 [34 kDa], -3 [44&40 kDa], -4 [22 kDa], -5 [30 kDa], and -6) in pig ovary (Giudice, 1992; Grimes *et al.,* 1994; Shimasaki *et al.,* 1991), and two IGFBPs (bIGFBP-2 [34 kDa] and -3 [40&44 kDa]) in cattle ovaries (Echternkamp *et al.,* 1994). In addition, 29-27 and 22 kDa bands were detected but not identified by immunoprecipitation in bovine FF (Echternkamp *et al.*, 1994).

At least three IGFBPs (-2, -4 and -5) have been shown to have antigonadotropic effects on ovarian FSH stimulation in humans, rats, pigs and cows. In humans, IGFBP-2 (Cataldo and Giudice, 1992; San Roman and Magoffin, 1993) and IGFBP-4 (Giudice *et al.,* 1993) levels increased in atretic follicles whereas, IGFBP-3 levels in dominant and atretic follicles decreased during folliculogenesis (San Roman and Magoffin, 1993). In rats, IGFBP-4 and -5 mRNAs levels are increased in granulosa cells of atretic follicles but are not expressed or are expressed at very low levels in granulosa cells of dominant follicles (Erickson *et al.*, 1992a,b). In pigs, IGFBP-2 (Howard and Ford, 1992), IGFBP-4 and -5 (Grimes *et al.*, 1994) are present predominantly in atretic and in small immature follicles. Similar results have been described for cattle, where FF IGFBP-2, and amounts of two unidentified IGFBPs (29-27 kDa and 22 kDa) were inversely related to FF E_2 concentrations (Echternkamp *et al.*, 1994). Furthermore, IGFBPs levels were higher in FF from bovine follicles that contained oocytes with reduced developmental potential and in follicles that contained oocyte cumulus complexes that failed to develop to the blastocyst stage (Hazeleger *et al.*, 1993).

The gene encoding IGFBP-2 is expressed strongly in the rat ovary; mRNA is present in theca-interstitial cells and surface epithelium. The message is not present in granulosa cells (healthy or atretic follicles), theca externa, corpora lutea and stromal tissue (Erickson *et al.*, 1994). In the pig, IGFBP-2 mRNA is expressed in granulosa cells and theca cells (Samaras *et al.*, 1992) whereas in humans, IGFBP-2 mRNA is expressed in granulosa and theca cells of follicles at all stages of development (Zhou and Bondy, 1993). In cattle, although various studies (Echternkamp *et al.*, 1994; Stanko *et al.*, 1994) have been done to identify the different IGFBPs present in follicular fluid, mRNA studies to quantitate gene expression of IGFBP-2 and/or other IGFBPs are lacking.

In summary, IGF-I and -II and IGFBPs are produced in ovarian follicles throughout folliculogenesis. Insulin-like growth factor-I plays an important role in folliculogenesis by synergizing with FSH in stimulating granulosa cells to differentiate and proliferate. The IGFBPs appear to play a pivotal role in binding to FF IGF and thereby suppressing IGF actions in autocrine and paracrine loops. Although several studies have been done in the past to describe changes in IGF-I and -II and IGFBPs during the follicular phase of the estrous cycle, a detailed study that compares IGF-I and -II and IGFBPs changes in dominant vs subordinate follicles as well as in E_2 active vs atretic follicles of cattle is lacking. In Chapter 3, an experimental model to obtain first wave dominant and subordinate follicles at defined stages of development and in a state of hormonally altered follicle turnover was described. The use of such a model would permit us to undertake a detailed study of changes in the amounts and proportions of different IGFBPs in follicular fluid of E_2 active, transitional E_2 active, atretic, and E_2 active sustained dominant follicles and the cohort of respective subordinate follicles. Thus, the objective of this chapter was to measure IGF-I and IGF-II concentrations, qualitative and quantitative changes in the different IGFBPs present in follicular fluid, and mRNA levels for IGFBP-2 in first wave dominant and subordinate follicles at different stages of development in cattle utilizing the model described in Chapter 3. In addition, the interrelationships between IGF concentrations, IGFBPs amounts in follicular fluid and follicular mRNA levels for IGFBP-2 were characterized in dominant follicles to determine their interrelationships and potential involvement in regulating dominance and atresia.

Material and Methods

The reader is referred to the material and methods section of Chapter 3 for a detailed description of the experimental animals and reproductive management, ultrasonographic examination and blood collection, tissue collection and handling, steroid hormone RIA in FF, RNA isolation, Northern and dot blot analyses, phosphor screen autoradiography and statitistical analyses utilized in the present section.

Total Protein Concentrations in Follicular Fluid

Total protein concentrations (mg/ml) in FF of DF (n=43) and SF (n=78) were measured in duplicate by the biocinchoninic acid (BCA; Pierce, Rockford, IL) protein assay as described previously (Smith *et al.,*1985), with bovine serum albumin as the standard.

IGF-I and IGF-II Assay in Follicular Fluid

Insulin-like growth factor binding proteins were removed from FF of DF (n=42) and SF (n=39) using Sep-Pak Plus C_{18} cartridges (Waters, Milford, MA) following the original procedure described for serum samples by Lee *et al.* (1991; Appendix 7). Briefly, 0.2 ml of FF from individual DF, SF or a pool of cohort SF were acidified with 1.3 ml of 1% aqueous trifluoroacetic acid (TEA) for 10 min at room temperature (RT). The cartridges were preconditioned by sequential washes of 100% acetonitrile, deionized water and 0.1% aqueous TEA. Then the acidified FF (1.5 ml) was loaded into the cartridge, and flushed with three 1 ml volumes of 0.1% aqueous TEA to elute IGFBPs. The retentate which contained free IGF-I and -II was eluted in 2 ml of acetonitrile containing 0.1% TEA. The acetonitrile eluate was air-dried in a 37°C water bath and the residue solubilized in 4 ml of RIA buffer (30 mM phosphate [pH 7.5], 0.02% protamine sulfate, 10 mM EDTA, 0.05% Tween-20 and 0.02% sodium azide). The solution was centrifuged at 3,000 rpm for 30 min, and 20 μl (1 μl native FF equivalent) was used for RIA.

The effectiveness of the Sep-Pak procedure to remove IGFBPs from FF was assessed by measuring the IGF binding activity before and after Sep-Pak chromatography (Lee *et al.,* 1991) of a pool of FF. Briefly, the initial pass-through, the three-1 ml volumes washes of 0.1% TEA (which contains IGFBPs) and the acetonitrile 0.1% TEA eluates (which contains IGF-I and -II) from FF pool samples were reconstituted in RIA buffer. Graded doses (2, 4, 8, 16, 32, 48, 64, 80, 96, 112 and 128 μl) of these eluates and raw FF were incubated with 50,000 cpm ^{125}I IGF-II for 2 hrs at RT in a total volume of 0.4 ml. The incubation mixtures received an equal volume of cold 1% charcoal suspension in RIA buffer and were incubated for 8 min at RT. Then the samples were centrifuged at 3,000 rpm for 30 min, and 0.4 ml of supernatant was removed and counted.

One microgram of recombinant human [^{59}Thr] IGF-I and IGF-II (AmGen Biologicals, Thousand Oaks, Ca) were iodinated (Appendix 7) to a specific activity of 150-300 μCi/μg protein, using 1 mCi Na^{125}I (Amersham, Arlington Heights, IL) and 2.5 μg Iodogen (Pierce, Rockford, IL). Iodinated IGF were purified on a Sephadex G-50 (fine) column and aliquots were stored at -20°C until used. A FF Sep-Pak aliquot (IGF-I=20 μl) was incubated with IGF-I rabbit antiserum (Appendix 7; UBK 487, National Hormone and Pituitary Program; final dilution 1:20,000) and 15,000 to 20,000 cpm ^{125}I IGF-I in RIA buffer (total volume: 0.5 ml) for 18 hrs at 4°C. Then, 0.1 ml of 1:10 diluted sheep antiserum to rabbit γ-globulin was added and the mixture incubated for 1 hr. This was followed by addition of 0.1 ml of 1:100 diluted normal rabbit serum and incubation for 1 additional hour at 4 °C. After the addition of 1 ml of RIA buffer, tubes were centrifuged at 3,000 rpm for 30 min at 4 °C. The supernatant was aspirated, pellet counted for radioactivity, and the IGF-I concentrations expressed in ng/ml of FF. For the IGF-II assay (Appendix 7), 10 μl (0.5 μl of native FF equivalent) of the same Sep-Pak separated FF aliquot was incubated with 1:20,000 mouse monoclonal immunoglobulin G (IgG) against rat (r) IGF-II (Amano Co. Troy, VA) and 15,000 to 20,000 cpm ^{125}I IGF-I in the same RIA buffer (total volume: 0.5 ml) under the same incubation conditions as those described for the IGF-I RIA. A goat antiserum to mouse IgG and normal mouse serum (Sigma Chemical Co., St. Louis, MI) were used to precipitate the antigen antibody complexes following incubation conditions similar to those described for the IGF-I RIA (Appendix 7). The supernatant was aspirated, the pellet counted, and the IGF-II concentrations expressed in ng/ml of FF.

The validation procedure for IGF-I and IGF-II RIAs was completed using two different methods: 1) parallelism and 2) recovery of added mass. Parallelism was tested by comparing the slopes of an inhibition curve caused by adding increasing amounts of sample (2.5, 5, 10, 20, 70, 120, 170, 220, 270 and 320 μl of FF pool) to the slope of the standard curve. Accuracy of the RIA procedure was determined by measuring known quantities of exogenous IGF-I or IGF-II previously added to a pool of extracted bFF. Added masses achieved final concentrations of 40, 80, 160, 320 and 640 pg/tube. These FF pool samples were assayed in volumes of 20 μl (IGF-I) and 10 μl (IGF-II). The minimum detectable concentration for the IGF-I assay was 5.76 ng/ml and for the IGF-II assay was 10.65 ng/ml. The intra- and inter-assay coefficients of variation were 5.12% and 7.56% (IGF-I) and 4.83% and 6.89% (IGF-II), respectively.

Ligand Blot Analysis of IGFBPs in Follicular Fluid

Twenty-five micrograms of FF protein (DF, n=43; SF, n=71) were mixed with distilled water (final volume 20 μl) and 20 μl of 2X sample buffer (1X: 62.5 mM Tris, pH 6.8; 5% SDS; 10% sucrose; 0.02% bromophenol blue). After boiling for 3 min, the mixture, FF pool (n=2) and protein molecular weight standards (Bio-Rad, Richmond, CA) were loaded onto a 3% stacking gel, and electrophoresed (25 mA) through a 12.5% polyacrylamide gel under non-reducing conditions (Laemmli, 1970; Appendix 8). Each electrophoresis run (n=5) contained two gels. After electrophoresis (n=10 gels), proteins were transferred onto a nitrocellulose membrane (0.2 μm, Schleicher and Schuell, Inc., Keene, NH) overnight at 200 mA at 4°C in Towbin buffer (25 mM Tris, pH 8.3; 192 mM glycine; 20% methanol) using a Hoefer (San Francisco, CA) electrophoresis transfer unit. Ligand blotting was performed following the procedure described by Lee *et al.* (1991; Appendix 8). After electrotransfer, the nitrocellulose membranes were air-dried, washed with TBS (10 mM Tris, pH 7.4; 150 mM NaCl and 0.05% sodium azide), and incubated with 1% non-fat dry milk in TBS on a rocking platform for 1 hr at RT. The membranes were washed with TBS containing 0.1% Tween-20 for 10 min and then incubated with I^{125} IGF-II (0.6 x 10^6 cpm/100 cm^2 membrane) in TBS containing 0.1% Tween-20 and 1% BSA (Sigma Chemical Co., St. Louis, MO) in heat sealable pouches (Kapak®, St. Louis Park, MN) for 20-24 hrs at 4°C on rocking platform. After rinsing the membranes with TBS, they were washed twice with TBS containing 0.1% Tween-20, and washed twice with TBS on a rocking platform for 15 min per wash. Membranes were air-dried, and subjected to autoradiography and phosphor screen autoradiography.

Immunoprecipitation Analysis of IGFBPs in Follicular Fluid

Two hundred-fifty micrograms of FF protein (pool of DF, pool of SF; Appendix 9) were incubated with either no antiserum against IGFBPs (positive control), with 5 μl of antiserum to IGFBP-2 (rabbit anti bovine IGFBP-2; Upstate Biotechnology, Inc., Lake Placid NY), IGFBP-3 (rabbit anti human rIGFBP-3; Upstate Biotechnology, Inc., Lake Placid NY), IGFBP-4 (rabbit anti human IGFBP-4; Upstate Biotechnology, Inc., Lake Placid NY) IGFBP-5 (rabbit anti human rIGFBP-5; Austral Biologicals, San Francisco, CA), or IGFBP-6 (rabbit anti human rIGFBP-6; Austral Biologicals, San Francisco, CA) or with 5 μl of normal rabbit serum (negative control) and 85 μl 50 mM sodium phosphate, pH 7.4, overnight at 4°C. The mixture then was added to 150 μl of washed 0.1% Protein A-Sepharose beads (Pharmacia, Piscataway, NJ) along with 500 μl of 50 mM Tris, pH 7.4, and the mixtures incubated overnight at 4°C on a rotating shelf. After washing the insoluble Protein A-Sepharose beads three times with Tris buffer containing 0.5% Triton X-100, the pellet (immunoprecipitated product) was mixed with 50 μl of 2X SDS-gel sample buffer, boiled for 3 min and briefly centrifuged. The resolubilized mixture was subjected to Ligand Blotting (Appendix 8).

cDNA Probe

The cDNA that encodes rat IGFBP-2 (530 bp; Brown *et al.,* 1989) was a generous gift from Dr. M.M. Rechler (NIH, Bethesda, MD). To correct for potential unequal loading of the RNA and variations in transfer efficiency, hybridization to 28S rRNA (human [5028 bp DNA], Gonzalez *et al.*,1985; kindly provided by Dr. R. Schmickel) was also determined. The IGFBP-2 cDNA and the 28S rDNA were labeled with [α-^{32}P]deoxy-

CTP (ICN Radiochemicals, Irvine, CA) by use of Multiprime DNA Labeling System (Amersham Int., UK; Appendix 5). The labeled cDNA and rDNA probes were separated from unincorporated label using a Sephadex® G-50 gel filtration column (Pharmacia Biotechnology Inc., Piscataway, NJ).

Statistical Analyses

Data were analyzed by least squares analysis of variance using the General Linear Models Procedure (GLM; SAS®, 1988) as previously described in Chapter 3. In addition, a novel approach was followed to analyze the absolute amounts and the proportions of $_{\mathrm{LIGB}}$IGFBPS present in follicular fluid. The intensity (arbitrary units, AU) of each $_{\mathrm{LIGB}}$IGFBPs band detected in the two pools of FF present in each gel were analyzed with a mathematical model that included the main effects of gel and run, and the interaction of gel by run. The least square mean of each band within gel and within run were later used as a covariate for the corresponding bands in all samples within the same gel and run. The mathematical model used for the second statistical analysis was the same as that described in Chapter 3.

Results

Progesterone and Estradiol Concentrations in Follicular Fluid

The PERT was higher in SF of intact (period 1) cows compared to unilateral ovariectomized (period 2) cows (46.79±5.50 vs 9.10±3.97; Fig. 6-1, left) , but were not different for DF of either group (7.09±2.78 vs 5.96±2.87; period by follicle interaction, P<.01). In addition, dominant and subordinate follicles exhibited distinct changes among experimental groups (experimental group by follicle interaction, P<.03; Fig. 6-1, right). A separate analysis that included only DF indicated that D5 (E_2 active) and D8 (E_2 active-transitional) follicles did not differ in PERT (0.10±6.39 vs 0.28±7.38, P<.99; Fig. 6-1, right), but D12+I E_2 active-sustained DF had lower PERT compared to D12-I atretic DF (2.23±7.00 vs 23.48±7.30, P<.05; Fig. 6-1, right). A similar approach was followed with the SF analysis. In D12+I SF, the PERT was higher than in D12-I SF (49.65±18.88 vs 3.60±15.51, P<.08; Fig. 6-1, right). Table 6-1 depicts the frequency of follicles (DF vs SF) within each experimental group (D5, D8, D12-I and D12+I) with PERT <1 or ≥1 (experimental group by follicle interaction, P<.01). Subordinate follicles had a higher frequency of follicles with PERT ≥1 than DF (27/44 vs 11/44, P<.04; Table 6-1). Whereas the frequencies of SF with PERT ≥ 1 were not different between D12-I and D12+I (6/11 vs 4/11, P<.25; Table 6-1), the frequency of D5 SF with PERT ≥1 was strikingly higher to that observed in D8 SF (12/12 vs 5/10, P<.04; Table 6-1). The frequency of DF with PERT ≥1 in D5 (0/12) and D8 (1/10) DF was lower (P<.01; Table 6-1) than the frequency of DF with PERT ≥1 in D12-I (9/11) and D12+I (1/11) DF was lower (P<.01; Table 6-1). Although, no frequency differences were observed between D5 and D8 DF (0/12 vs 1/10, P<.54; Table 6-1), a very striking difference was observed between D12-I and D12+I (9/11 vs 1/10, P<.01; Table 6-1).

Total Protein Concentrations in Follicular Fluid

Total protein concentrations (mg/ml) were similar among experimental groups (D5:106.03±5.81, D8:92.35±6.21, D12-I:99.48±6.73 and D12+I:102.34±5.55;

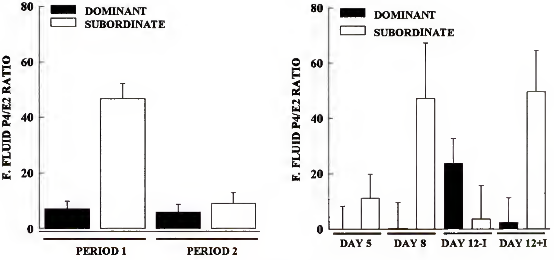

Figure 6-1. Follicular fluid P_4:E_2 ratio in first wave DF and SF of dairy cows. Data represent least square means (±SEM) of follicular fluid PERT in two periods (1=intact, 2=unilateral ovariectomized; left) or in four experimental groups (D5, D8, D12-I and D12+I; right).

$P<.45$) and between dominant and subordinate follicles (105.46±4.69 vs 94.64±4.69; $P<.10$; Fig. 6-2, left). Furthermore, separate analysis of dominant and subordinate follicles total protein concentrations did not detect differences among experimental groups (DF, $P<.28$; SF, $P<.78$; Fig. 6-2, left). Conversely, total protein contents (mg/follicle) were different among experimental groups and between dominant and subordinate follicles (experimental group by follicle interaction; $P<.01$; Fig. 6-2, right). Separate analysis ofDF, indicated that D5 and D8 DF, had lower total protein content compared to D12-I

Table 6-1. Frequency of follicles (dominant and subordinates) with P_4:E_2 ratio <1 or ≥1 in the four experimental groups studied (day 5, E_2 active; D8 transitional E_2 active; D12-I atretic and D12+I E_2 active-sustained).

	Day 5[a]		Day 8[b]		Day 12-I[c]		Day 12+I[d]	
P_4:E_2 ratio	<1	≥1	<1	≥1	<1	≥1	<1	≥1
DF[e]	12	0	9	1	2	9	10	1
SF[f]	0	12	5	5	5	6	7	4

[a] Day 5 E_2 active experimental group,
[b] Day 8 transitional E_2 active experimental group,
[c] Day 12-I atretic experimental group,
[d] Day 12+I E_2 active-sustained experimental group,
[e] Dominant follicle, [f] Subordinate follicle,
P_4:E_2 ratio orthogonal contrasts:

Experimental group by follicle interaction (P<.01);

DF, Experimental group (P<.01), D5&D8 vs D12-I&D12+I (P<.01), D5 vs D8 (P<.54), D12-I vs D12+I (P<.01);

SF, Experimental group (P<.03), D5&D8 vs D12-I&D12+I (P<.08), D5 vs D8 (P<.04), D12-I vs D12+I (P<.25).

and D12+I DF (222.40±50.10 vs 339.85±48.79; P<.03; Fig. 6-2, right). Furthermore, D5 and D8 DF had similar total protein content (189.72±48.79 vs 255.09±51.42; P<.24; Fig. 6-2, right) whereas D12-I had strikingly lower total protein content compared to D12+I DF (214.42 vs 465.28±48.79; P<.01, Fig. 6-2, right). No significant differences in total protein content were detected among experimental groups of SF (P<.17; Fig. 6-2, right).

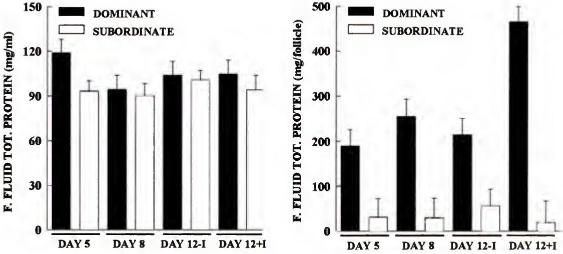

Figure 6-2. Total protein concentrations (mg/ml; left) and total protein content (mg/follicle; right) in follicular fluid from first wave DF and SF of dairy cows.

IGF-I and IGF-II Concentrations in Follicular Fluid

Follicular fluid IGFBPs were removed almost totally by acidification and chromatography on a Sep-Pak cartridge. Small proportions of FF IGF-binding activity were found in the Sep-Pak pass-through fraction (5.5%), and negligible proportions of FF IGF-binding activity were found in the 0.1% TFA (<1%) and acetonitrile 0.1% TFA (<1%) eluates (Fig. 6-3). Conversely, native FF had a high proportion of IGF-specific binding activity (>45%, Fig. 6-3). The separation efficiency of the Sep-Pak cartridge procedure was ≈50%, as estimated from recovery of radiolabeled IGF added to FF pools 2 hrs before separation; therefore IGF-I and IGF-II values were adjusted accordingly. Recovery of

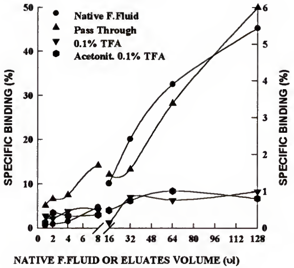

Figure 6-3. IGF-binding activity in Sep-Pak eluates of acidified bovine follicular fluid measured by charcol method.

added mass vs measured concentrations of IGF-I was described by linear regression ($Y=49.89+0.92X$; $R^2=0.99$; Fig 6-4, left). Recovery of added mass vs measured concentrations of IGF-II also was described by linear regression ($Y=63.55+0.76X$; $R^2=0.95$; Fig 6-5, left). Addition of increasing volumes of bovine FF pool demonstrated parallelism with IGF-I (homogeneity of regression, $P<.23$; Fig. 6-4, right) and IGF-II standard curves (homogeneity of regression, $P<.41$; Fig. 6-5, right). The minimum detectable concentration for the IGF-I RIA was 5.76 ng/ml and for the IGF-II RIA was 10.65 ng/ml. The intra- and inter-assay coefficients of variation were 5.12% and 7.56% (IGF-I) and 4.83% and 6.89% (IGF-II), respectively.

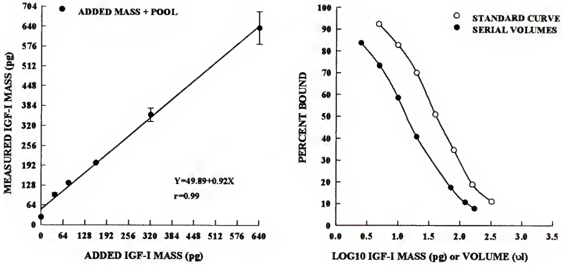

Figure 6-4 Recovery of added mass of IGF-I (Left) and RIA displacement of [^{125}I]IGF-I (Right).

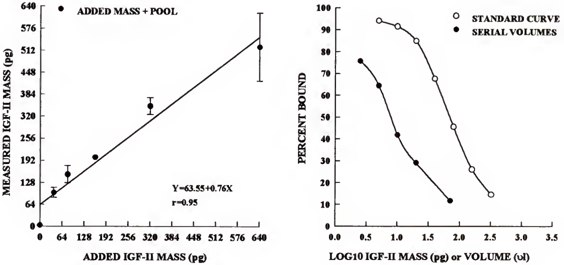

Figure 6-5. Recovery of added mass of IGF-II (Left) and RIA displacement of [^{125}I]IGF-II (Right).

Follicular fluid IGF-I concentrations (ng/ml) were similar among experimental groups (D5:113.75±9.41, D8:99.38±14.38, D12-I:115.39±10.33 and D12+I: 90.52±14.01; $P<.49$) and between dominant and subordinate follicles (111.13±6.91 vs 98.40±17.59; $P<.46$; Fig. 6-6, left). Within DF, no experimental group differences were detected ($P<.42$; Fig. 6-6, left). Similar results were observed for SF IGF-I ($P<.32$; Fig. 6-6, left). Follicular fluid IGF-II concentrations (ng/ml), were similar among SF but changed across DF of the experimental groups (experimental group by follicle interaction, $P<.05$; Fig. 6-6, right). Separate analyses of DF indicated that IGF-II concentrations differed between D5 and D8 DF (163.77±10.79 vs 92.68±11.37, $P<.01$; Fig. 6-6, right), and that IGF-II concentrations between D12-I and D12+I were similar (125.02 vs 120.74±11.37, $P<.78$; Fig. 6-6, right). Similar analysis was performed between SF, and no differences in IGF-II concentrations were detected ($P<.76$; Fig. 6-6, right).

Ligand Blot Analysis of IGFBPs Content in Follicular Fluid

Follicular fluid IGF-binding protein activity was characterized by ligand blotting using $[^{125}I]$IGF-II. A ligand blot with a representative set of first wave D5, D8, D12-I and D12+I dominant and subordinate follicles is presented in Fig. 6-7. In decreasing M_r (kDa) order, six bands were detected by autoradiography: 49, 43, 35, 30, 28 and 22 kDa (Fig. 6-7).

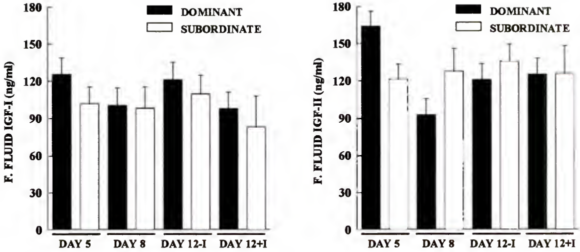

Figure 6-6. Follicular fluid IGF-I (left) and IGF-II (right) concentrations (ng/ml) in first wave DF and SF of dairy cows.

Immunoprecipitation of IGFBPs in Follicular Fluid

Immunoprecipitation analysis of a pool of follicular fluids representative of first wave dominant and subordinate follicles is depicted in Figure 6-8. In decreasing M_r order, 6 protein bands were identified: 49, 43, 35, 30, 28 and 22 kDa. The 35 kDa band corresponded to IGFBP-2, the 49 and 43 kDa bands corresponded to IGFBP-3, the 28 and 22 kDa bands corresponded to IGFBP-4 and the 30 kDa band corresponded to IGFBP-5. In addition, no immunoprecipitated product was detected at 22 kDa (human) or 26.5 kDa (rat) with the antiserum against IGFBP-6 (Fig. 6-8) and IGFBP-1 was not examined (antibody for bovine IGFBP-1 not available).

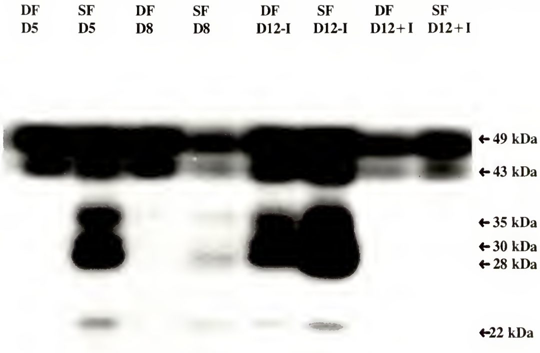

Figure 6-7. Ligand blot analysis of follicular fluid from representative first wave D5, D8, D12-I and D12+I dominant and subordinate follicles. Twenty-five μg of total protein were loaded per follicle/lane. Nitrocellulose membranes were incubated with radiolabeled ^{125}I-IGF-II. In decreasing M_r order, 6 bands were detected: 49, 43, 35, 30, 28 and 22 kDa.

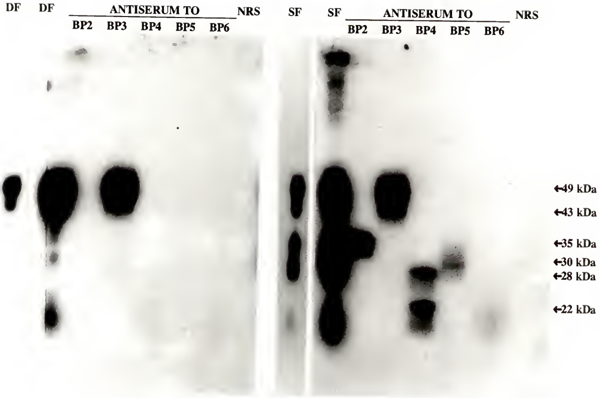

Figure 6-8. Immunoprecipitation analysis of follicular fluid from a pool of representative first wave dominant (left) and subordinate (right) follicles. Two hundred and fifty micrograms of total protein (pool of DF, pool of SF) were incubated with either no antiserum against IGFBPs (positive control), with 5 μl of antiserum to IGFBP-2, -3, -4, -5, -6 or with 5 μl of normal rabbit serum (negative control) and 85 μl 50 mM sodium phosphate, pH 7.4, overnight at 4°C. The mixture then was added to 150 μl of washed Protein A-Sepharose beads along with 500 μl of 50 mM Tris, pH 7.4, and the mixture was incubated overnight at 4°C on rotating shelf. After washing the insoluble Protein A-Sepharose beads 3X with Tris buffer containing 0.5% Triton X-100, the pellet was mixed with 50 μl of 2X SDS-gel sample buffer, boiled for 3 min, centrifuged for 5 sec and subjected to Ligand Blotting. Nitrocellulose membranes were incubated with radiolabeled ^{125}I-IGF-II. In decreasing M_r order, 4 forms were detected: IGFBP-3 (49 and 43), IGFBP-2 (35), IGFBP-5 (30) and IGFBP-4 (28 and 22 kDa).

IGFBPs Content in Follicular Fluid

Total ${}_{LIGB}$IGFBPs intensity (sum of arbitrary units [AU] for all IGFBPs [-2, -3, -4 and -5] for each individual follicle [25 μg of FF protein/follicle]) had different patterns of change for DF and SF (368.57 ± 82.6 vs 624.14 ± 71.44; $P<.04$; Fig. 6-9). In contrast, no differences in total ${}_{LIGB}$IGFBPs intensity were detected among experimental groups($P<.51$; Fig. 6-9).Separate analysis of DF, indicated that D12-I DF had higher total ${}_{LIGB}$IGFBPs intensity compared to D12+I DF (530.62 vs 287.68 ± 84.83 AU, $P<.03$; Fig. 6-9) and that D5 and D8 DF had similar total ${}_{LIGB}$IGFBPs intensities (306.33 ± 77.44 vs 421.65 ± 89.42 AU, $P<.13$; Fig. 6-9).

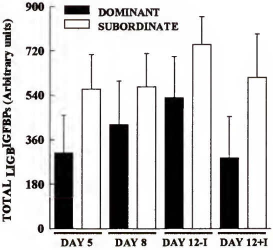

Figure 6-9. Least square means (±SEM) of total ${}_{LIGB}$IGFBPs (sum of arbitrary units for all forms [49 and 43 kDa: IGFBP-3; 35 kDa:IGFBP-2; 30 kDa:IGFBP-5; 28 and 22 kDa:IGFBP-4] of IGFBPs/follicle) in first wave DF and SF of non-lactating dairy cows.

Subordinate follicles also had similar total $_{LIGB}$IGFBPs intensities (AU) among experimental groups (P<.88; Fig. 6-9).

Whereas IGFBP-3 followed the same pattern of change in DF and SF described for total $_{LIGB}$IGFBPs intensity, the three remaining IGFBPs detected in bovine FF (IGFBP-2, -4 and -5) exhibited a different pattern of change in DF and SF compared to the total $_{LIGB}$IGFBPs and $_{LIGB}$IGFBP-3 intensities (Fig. 6-9; Fig. 6-10; IGFBP-2, top-left; IGFBP-3, top-right; IGFBP-4, bottom-left; IGFBP-5, bottom right). The $_{LIGB}$IGFBPs intensities for IGFBP-2, -4 and -5 were higher in SF compared to DF ([IGFBP-2, 107.17±12.17 vs 53.47±14.08 AU, P<.01; Fig. 6-10, top-left], [IGFBP-4, 169.74±24.41 vs 53.27±28.25 AU, P<.01; Fig. 6-10, bottom-left] and [IGFBP-5, 134.50±25.05 vs 42.42±28.98 AU, P<.01; Fig. 6-10, bottom-right]). Separate analysis of DF using orthogonal contrasts detected differences in $_{LIGB}$IGFBPs intensity between D12-I and D12+I DF ([IGFBP-2, 85.93 vs 46.77±17.95 AU, P<.05; Fig. 6-10, top-left], [IGFBP-4, 99.13 vs 31.83±19.44 AU, P<.02; Fig. 6-10, bottom-left] and [IGFBP-5, 65.76 vs 23.85±18.60 AU, P<.05; Fig. 6-10, bottom-right]). However, within SF, no differences among experimental groups were detected ([IGFBP-2, P<.96; Fig. 6-10, top-left], [IGFBP-4, P<.80; Fig. 6-10, bottom-left] and [IGFBP-5, P<.90; Fig. 6-10, bottom-left]).

The IGFBP-3 had quite a different pattern of $_{LIGB}$IGFBP intensity as compared to the other three IGFBPs (-2, -4, -5; depicted in Fig 6-10, top-right). No differences in$_{LIGB}$IGFBP-3 intensities were detected either among experimental groups (D5:204.35±30.92 AU; D8:231.45±33.11 AU; D12-I:273.61±30.12 AU;

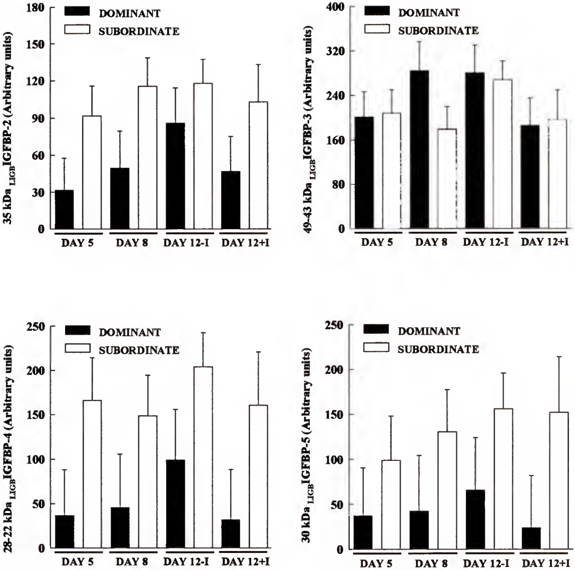

Figure 6-10. Least square means (±SEM) of ${}_{\text{LIGB}}$IGFBP intensity (arbitrary units of IGFBP-2 [35 kDa, top-left], IGFBP-3 [49 and 43 kDa, top-right], IGFBP-4 [28 and 22 kDa, bottom-left] and IGFBP-5 [30 kDa, bottom-right] per follicle) in first wave DF and SF of non-lactating dairy cows.

D12+I:190.81±36.22 AU; P<.26; Fig. 6-10, top right) or between dominant and subordinate follicles (237.39±24.72 AU vs 212.72±21.36 AU, P<.40; Fig. 6-10, top-right). Separate analysis of DF indicated that there were no differences in ${}_{\mathrm{LIGB}}$IGFBPs among experimental groups (DF P<.33; SF P<.44; Fig. 6-10, top-right).

The proportion of each binding protein to the total ${}_{\mathrm{LIGB}}$IGFBPs in FF for DF are presented in the left panel of Fig. 6-11, whereas those for SF are presented in the right panel of Fig. 6-11. For the four IGFBPs studied, striking differences in the proportion of individual IGFBPs were detected between dominant and subordinate follicles ([IGFBP-2, 12.67±1.26% vs 17.64±1.09%, P<.01] [IGFBP-3, 65.34±3.35% vs 42.50±2.89%, P<.01], [IGFBP-4, 12.31±1.46% vs 23.23±1.26%, P<.01] and [IGFBP-5, 9.66±1.48% vs 16.71± 1.28%, P<.01] Fig. 6-11, left&right). Within DF, the ${}_{\mathrm{LIGB}}$IGFBPs intensity of IGFBP-4 was higher in D12-I DF compared to D12+I DF (11.92 vs 17.46±2.02%, P<.06; Fig. 6-11). However, the proportion of ${}_{\mathrm{LIGB}}$IGFBPs intensity was similar across experimental groups for DF (IGFBP-2, P<.10; IGFBP-3, P<.37; IGFBP-5, P<.51; Fig. 6-10, left) and for SF (IGFBP-2, P<.39; IGFBP-3, P<.53; IGFBP-4, P<.25; IGFBP-5, P<.28; Fig. 6-11, right).

IGFBP-2 mRNA Content in Follicle Walls

To take into account potential differences due to unequal loading and transfer efficiency among samples, each individual follicle IGFBP-2 mRNA signal was adjusted for its corresponding 28S rRNA level. Pictures of representative sets of dot blots for IGFBP-2 mRNA are shown in Fig. 6-12. The mRNA levels for IGFBP-2 were higher in follicles of cows that were unilaterally ovariectomized (period 2) compared to those from

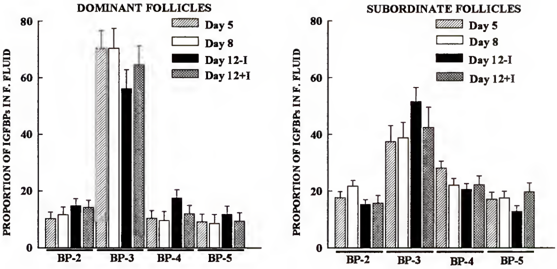

Figure 6-11. Least square means (±SEM) of proportion of $_{LIGB}$IGFBP intensity (arbitrary units of IGFBP-2 [35 kDa], IGFBP-3 [49 and 43 kDa], IGFBP-4 [28 and 22 kDa] and IGFBP-5 [30 kDa] per follicle) in first wave DF (left) and SF (right) of non-lactating dairy cows.

intact (period 1) cows (101.69±5.67 vs 63.89± 5.34 AU, P<.01; Fig. 6-13, left). However, no interaction of period by experimental group (P<.49) or with follicle were detected (P<.91). Levels of IGFBP-2 mRNA had a different pattern of expression among experimental groups and between follicles (experimental group by follicle interaction, P<.07; Fig. 6-13, right). In D5 and D8 DF, expression of IGFBP-2 mRNA was lower compared to D12-I and D12+I DF (82.69±9.84 vs 104.00±10.26 AU, P<.05; Fig. 6-13, right). In contrast no differences were detected between D5 and D8 (88.16±8.98 vs 77.23±10.71 AU, P<.45; Fig. 6-12, right) nor between D12-I and D12+I DF (97.70±10.84 vs 110.30±9.68 AU, P<.40; Fig. 6-13, right). Moreover, no differences

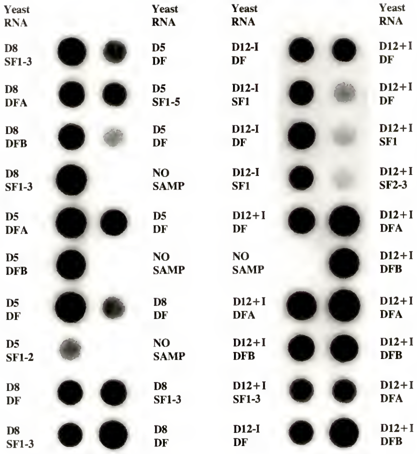

Figure 6-12. Dot blot analysis of IGFBP-2. Five μg of total RNA extracted from first wave DF and SF at D5, D8, D12-I and D12+I were loaded per sample. Hybridization was done with [α-^{32}P]deoxy-CTP radiolabeled IGFBP-2 cDNA fragment.

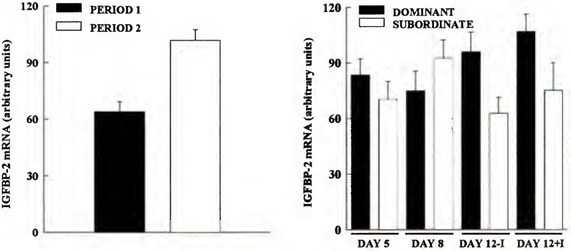

Figure 6-13. Least square means (±SEM) of hybridization intensity (arbitrary units) of IGFBP-2 cDNA to mRNA from first wave DF and SF of non-lactating dairy cows.

in levels of IGFBP-2 mRNA were detected among experimental groups for SF ($P<.15$; Fig. 6-13, right).

Correlation and Partial Correlation Analyses

In Table 6-2 are depicted the simple (top-right) and partial correlations (bottom-left) coefficients between concentrations of IGF-I and IGF-II (ng/ml), contents of IGFBP-2, -3, -4 and -5 ($_{LIGB}$IGFBPs, arbitrary units/10 μg total protein) and steady state mRNA levels (arbitrary units/5 μg of total RNA) of IGFBP-2 in DF. Two salient points were observed: 1) IGF-I was correlated with IGF-II ($r=0.50$, $P<0.01$) and with IGFBP-3 ($r=0.43$, $P<0.01$) and 2) all IGFBPs were highly associated with each other ($r>.39$, $P<0.01$). Partial correlations between the same set of variables are depicted in Table 6-2 (bottom-left). These correlations have been adjusted for the effects of experimental group, period, experimental group by period and for cow variation. Three important correlations were observed: 1) IGF-I was correlated to IGF-II ($r=0.65$, $P<0.01$), 2) IGF-II was correlated with IGFBP-2 ($r=0.76$, $P<0.01$) and 3) the three low M_r IGFBPs (-2, -4 and -5) were highly correlated with each other ($r>0.84$, $P<0.01$) but were not correlated with IGFBP-3 ($r>-0.09$, $P<0.54$).

Table 6-2. Simple (top right) and partial (bottom left) correlations between concentrations of IGF-I, IGF-II (ng/ml), amounts of 35 kDa IGFBP-2, 49-43 kDa IGFBP-3, 28-22 kDa IGFBP-4 and 30 kDa IGFBP-5 (${}_{\text{LIGB}}$IGFBPs; arbitrary units/25μg total protein) and steady state mRNA levels (arbitrary units/5μg total RNA) of IGFBP-2 in dominant follicles. Within parenthesis are the P values for each coefficient.

	IGF-I[a]	IGF-II[b]	IGFBP2[c]	IGFBP3[d]	IGFBP4[e]	IGFBP5[f]	MBP2[g]
IGF-I		0.50 (<0.01)	0.16 (0.29)	0.43 (<0.01)	0.12 (0.41)	0.10 (0.52)	0.01 (0.98)
IGF-II	0.65 (0.01)		0.05 (0.72)	0.03 (0.81)	-0.07 (0.62)	-0.08 (0.57)	-0.29 (0.06)
IGFBP2	0.38 (0.18)	0.76 (<0.01)		0.71 (<0.01)	0.89 (<0.01)	0.72 (<0.01)	0.12 (0.41)
IGFBP3	0.48 (0.09)	0.10 (0.73)	0.08 (0.79)		0.55 (<0.01)	0.39 (<0.01)	0.13 (0.38)
IGFBP4	0.13 (0.66)	0.48 (0.09)	0.89 (<0.01)	-0.09 (0.75)		0.91 (<0.01)	0.22 (0.15)
IGFBP5	0.11 (0.71)	0.44 (0.12)	0.84 (<0.01)	-0.17 (0.55)	0.97 (<0.01)		0.26 (0.08)
MBP2	-0.19 (0.52)	-0.17 (0.55)	-0.03 (0.92)	-0.18 (0.54)	-0.01 (0.99)	-0.03 (0.90)	

[a] IGF-I, [b] IGF-II,
[c] ${}_{\text{LIGB}}$IGFBP-2 (35 kDa), [d] ${}_{\text{LIGB}}$IGFBP-3 (49-43 kDa),
[e] ${}_{\text{LIGB}}$IGFBP-4 (28-22 kDa), [f] ${}_{\text{LIGB}}$IGFBP-5 (30 kDa),
[g] IGFBP-2 mRNA content.

Simple and partial correlations were calculated using IGF-I and IGF-II content per follicle (ng/follicle), contents of IGFBP-2, -3, -4 and -5 per follicle (arbitrary units/total protein/follicle) and IGFBP-2 mRNA contents per follicle (arbitrary units/total RNA/follicle). Partial correlations were adjusted for experimental group, follicle, period and cow variation whereas simple correlations were not. Simple correlations are depicted in the top-right of Table 6-3, whereas partial correlations are depicted in the bottom-left of Table 6-3. Insulin-like growth factor-I was correlated with IGF-II ($r=0.87$, $P<0.01$) and with IGFBP-3 ($r=0.64$, $P<0.01$), as described above, but also was correlated with IGFBP-2 ($r=0.39$, $P<0.01$) and IGFBP-2 mRNA levels ($r=0.43$, $P<0.01$). In addition, IGF-II was correlated with IGFBP-2 ($r=0.31$, $P=0.03$) and IGFBP-3 ($r=0.62$, $P<0.01$). The three low M_r IGFBPs (-2, -4 and -5) were highly correlated with each other ($r>0.78$, $P<0.01$) but were not correlated with IGFBP-3 ($r>0.08$, $P<0.30$). Although the same trends were observed with the partial correlations (Table 6-3, bottom-left), additional associations were detected. Insulin-like growth factor-II was correlated with IGF-I ($r=0.86$, $P<0.01$), with IGFBP-2 ($r=0.78$, $P<0.01$), with IGFBP-3 ($r=0.37$, $P=0.02$) and with IGFBP-4 ($r=0.56$, $P=0.04$). As above, all low M_r IGFBPs (2, -4 and -5) were highly correlated with each other ($r>0.85$, $P<0.01$).

Stepwise Regression Analysis and Standardized Regression Coefficients

Standardized regression coefficients calculated from stepwise regression analyses of FF contents of IGF-I and IGF-II (ng/follicle), contents of IGFBP-2, -3, -4 and -5 ($_{LIGB}$IGFBPs; arbitrary units/total protein/follicle) and IGFBP-2 mRNA content (arbitrary units/total RNA/follicle) in DF are depicted in Figure 6-14. Only SRC with

Table 6-3. Simple (top right) and partial (bottom left) correlations between contents of IGF-I, IGF-II (ng/follicle), contents of 35 kDa IGFBP-2, 49-43 kDa IGFBP-3, 28-22 kDa IGFBP-4 and 30 kDa IGFBP-5 ($_{LIGB}$IGFBPs; arbitrary units/follicle) and contents of IGFBP-2 mRNA (arbitrary units/follicle wall) in dominant follicles. Within parenthesis are the P values for each coefficient.

	IGF-I^{a}	IGF-II^{b}	IGFBP2^{c}	IGFBP3^{d}	IGFBP4^{e}	IGFBP5^{f}	MBP2^{g}
IGF-I		0.87 (<0.01)	0.39 (<0.01)	0.64 (<0.01)	0.26 (0.09)	0.23 (0.13)	0.43 (<0.01)
IGF-II	0.86 (<0.01)		0.31 (0.03)	0.62 (<0.01)	0.17 (0.27)	0.15 (0.33)	0.36 (0.01)
IGFBP2	0.49 (0.08)	0.78 (<0.01)		0.33 (0.02)	0.87 (<0.01)	0.78 (<0.01)	0.13 (0.41)
IGFBP3	0.29 (0.33)	0.37 (0.02)	0.16 (<0.59)		0.16 (0.29)	0.08 (0.58)	0.36 (0.01)
IGFBP4	0.25 (0.39)	0.56 (0.04)	0.92 (<0.01)	0.09 (0.75)		0.95 (<0.01)	0.09 (0.54)
IGFBP5	0.17 (0.56)	0.48 (0.09)	0.85 (<0.01)	-0.03 (0.91)	0.97 (<0.01)		0.03 (0.80)
MBP2	-0.12 (0.69)	-0.13 (0.65)	0.11 (0.97)	0.06 (0.98)	0.20 (0.50)	0.185 (0.53)	

^{a}IGF-I, ^{b}IGF-II,
$^{c}{}_{LIGB}$IGFBP-2 (35 kDa), $^{d}{}_{LIGB}$IGFBP-3 (49-43 kDa),
$^{e}{}_{LIGB}$IGFBP-4 (28-22 kDa), $^{f}{}_{LIGB}$IGFBP-5 (30 kDa),
^{g}IGFBP-2 mRNA content.

probability values ≤ 0.15 were included in the diagram depicted in Fig. 6-14. Bolded filled arrows represent strong and positive associations, whereas thin arrows represent associations that were considered relatively less or not important based upon relative magnitude of SRC. The SRC can be compared within variables but not among variables. For example, there are two arrows pointing to IGF-I content in FF: IGF-II (0.77) and IGFBP-3 (0.18); therefore these two SRC can be compared between each other, but the SRC 0.18 cannot be compared with the SRC 0.66 (IGF-I to IGFBP-3). Furthermore, a unit change in IGF-II content caused a 4.27 fold greater change in IGF-I content than did IGFBP-3. A more thorough explanation of the methodology used to develop these SRC and how they are interpreted is presented in Chapter 4. From inspection of Figure 6-14, it is clear that the variables studied are associated in two very distinctive groupings. One group represents the association of IGF-I with IGF-II (0.88) and with IGFBP-3 (0.66) and the reciprocal association of IGF-II with IGF-I (0.77; Fig. 6-14, right). The other group represents the association between the low M_r IGFBPs (2, 3 and 5; Fig. 6-14, left). In this last group, IGFBP-4 was strongly associated with IGFBP-2 (0.85) and with IGFBP-5 (0.96). A reciprocal strong association was also detected between IGFBP-5 and IGFBP-4 (0.70). Of the various variables examined, only IGF-I content in FF was associated with IGFBP-2 mRNA levels.

Discussion

In this study, changes in IGF-I and IGF-II concentrations, absolute levels and proportions of IGFBP-2, -3, -4, and -5, and mRNA levels of IGFBP-2 that occurred in first wave dominant and subordinate follicles during four very distinct stages of follicle development were characterized. Furthermore, these variables were studied by correlation, partial correlation and stepwise regression analyses to determine their interrelationships and potential involvement in the processes of dominance and atresia of dominant follicles.

Dominant and subordinate follicles were separated into two different populations based on the ratio of absolute concentrations of FF P_4 to E_2 (P_4:E_2 [PERT]; Grimes and

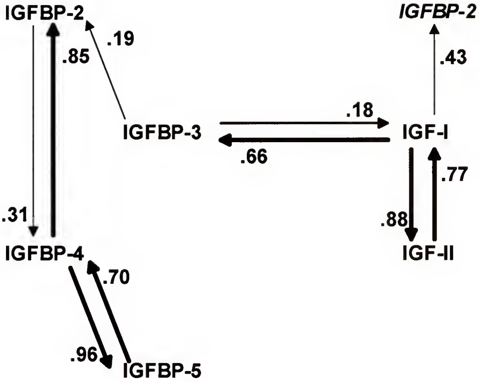

Figure 6-14. Standardized partial coefficients obtained from stepwise regression analysis of follicular fluid IGF-I and IGF-II content, $_{LIGB}$IGFBPs (IGFBP-2, 35 kDa; IGFBP-3, 49 and 43 kDa; IGFBP-4, 28 and 22 kDa; IGFBP-5, 30 kDa) content and IGFBP-2 mRNA content in whole dominant follicles.

Ireland, 1986; Grimes *et al.,* 1987; Ireland and Ireland, 1994; Martin *et al.,* 1991). Dominant and subordinate follicles with PERT >1 were classified as atretic whereas follicles with a PERT ≤ 1 were classified as E_2 active (Grimes and Ireland, 1986; Grimes *et al.,* 1987; Ireland *et al.*, 1994; Martin *et al.,* 1991). Nine out of twelve D12-I DF were atretic whereas none of the D5 DF were atretic and one out of ten and two out of eleven were atretic in D8 and D12+I DF, respectively (Table 6-1). Conversely for SF, all D5, half of D8, six out of eleven (D12-I) and four out of eleven (D12+I) SF were atretic (Table 6-1). This observation supports the view that most of SF in experimental groups with E_2 active DF were atretic whereas those present in E_2 inactive DF were from a new wave, and therefore were E_2 active. Several studies have used PERT alone (Ireland and Ireland, 1994b; Martin *et al.,* 1991) or with histological studies (i.e. E_2 active dominant follicles have <1% pycnotic cells, presence of mitotic figures, and integrity of at least 95% of granulosa cells against the basement membrane; Grimes and Ireland, 1986; Grimes *et al.,* 1987) to categorize ovarian follicles as atretic and E_2 active follicles. Although in this study, histological classification of individual follicles was not done because all of the follicle tissue was utilized for RNA extraction, the steroidal profiles of individual follicles (Chapters 3 and 4) and steroid ratios presented in Chapter 5 and here, followed the same pattern described previously by Ireland *et al.* (1994) and Martin *et al.* (1991). Dominant and subordinate follicles with a PERT >1 were classified accurately as atretic follicles (all D12-I DF, D5 [n=12], D8 [n=5], D12-I [n=6], and D12+I [n=4] SF; Table 6-1), whereas the remaining dominant and subordinate follicles were classified as E_2 active (all

D5, D8 [n=9] and D12+I [n=10] DF; D8 [n=5], D12-I [n=5] and D12+I [n=7] SF; Table 6-1). The differences in PERT observed between intact (period 1) and unilateral ovariectomized (period 2) cows (Fig. 6-1, left) agrees with FF P_4 profiles presented in Chapter 3 (Fig. 3-7, left). Hence these data support the idea that DF from unilaterally ovariectomized cows were more dominant during the first five days of the cycle compared to those from intact cows (i.e. dominance started 2 d earlier; Fig. 3-2, top-left and bottom). However after d 5 of the estrous cycle, DF from intact cows were more dominant and therefore induced a higher degree of atresia of SF which was expressed by higher levels of FF P_4 (Fig. 3-7, left) and smaller diameter at ovariectomy (Fig. 3-5, left).

The IGF-I concentrations in FF remained very much unchanged among experimental groups and between dominant and subordinate follicles (Fig. 6-6, left & right). Badinga *et al.* (1992) reported similar profiles between DF at d 5, 8 and 12 of the estrous cycle. In contrast, Echternkamp *et al.* (1990,1994) reported higher concentrations of IGF-I in FF of large E_2 active follicles compared to E_2 inactive and small follicles. The synergistic activity of IGF-I on FSH stimulation of granulosa cells is well documented (Adashi *et al.,* 1991; Giudice, 1992; Hammond *et al.,* 1991). Thus E_2 active follicles should have higher concentrations of IGF-I in FF to support their dominant and estrogen active role. However, this was not detected in the present study and evidence is now emerging to support the concept that IGFBPs may exert a pivotal role in this process by selectively binding to IGF-I present in FF and negating the potential trophic activity of IGF-I (Ui *et al.,*1989; Magoffin and Erickson, 1994). Therefore, the absolute concentrations of IGF-I in FF may remain constant but changes in the amounts and/or in the relative proportions of $_{LIGB}$IGFBPs present in FF, more IGF could be bound to the binding proteins and therefore less available for exerting their well documented effects on granulosa cells. The present study reports dramatic changes in the absolute amounts and in the proportions of at least three IGFBPs (2, 4 and 5) that are at low levels in E_2 active DF but are at high levels in atretic DF (Fig. 6-10, top-left, bottom-left and right; Fig 6-11, left & right). Thus, these results support the concept that increases in amounts and relative proportions of FF $_{LIGB}$IGFBP-2, -4 and -5 may be the mechanism by which the functional concentrations of IGF-I and IGF-II are regulated in atretic and subordinate follicles. Higher concentrations of these three IGFBPS in FF would bind more IGF-I and IGF-II and therefore reduce the concentrations of free IGF-I and IGF-II available in FF to exert their trophic activity on granulosa cells. Conversely, low concentrations of these three IGFBPS would augment concentrations of free IGF-I and IGF-II in FF and therefore induce potentially greater expression of P450 SCC (Magoffin *et al.*, 1990; Urban *et al.*, 1990) and P450 ARO message (Steinkamp *et al.*, 1988), and greater stimulation of androgen biosynthesis (Hernandez *et al.*, 1988), aromatase activity (Adashi *et al.*, 1985a,b; Bergh *et al.*, 1991; Erickson *et al.*, 1989, 1990), and inhibin production (Bicsak *et al.*, 1986). Indeed, the data reported here and in previous Chapters (3, 4 and 5) support these views. Day 12-I DF had higher absolute amounts and relative proportions of IGFBP-2, -4 and -5 in FF (Fig. 6-10, top-left, bottom-left&right) which very likely reduced free IGF-I and IGF-II concentrations in FF. In addition, these DF (D12-I) had very low concentrations of A_4 and E_2 in FF (Chapter 4; Fig 4-2), lower steady state levels of P450 17α-HYD and P450 ARO mRNA levels (Chapter 4; Fig 4-6, right; Fig. 4-9, right), and lower absolute amounts of $_{IM}$INH (Chapter 5; Fig. 5-4). Conversely, D5 DF had very high concentrations of FF A_4 and E_2 (Chapter 4; Fig 4-2), high steady state levels of P450 17α-HYD and P450 ARO mRNA levels (Chapter 4; Fig 4-6, right; Fig. 4-9, right), high absolute amounts of $_{IMB}$INH (Chapter 5; Fig. 5-4) and low absolute amounts of $_{LIGB}$IGFBP-2, -4 and -5. However, it is noteworthy to point out that the appearance of low M_r IGFBPs (-2, -4 and -5) is a gradual process that takes place during atresia of the first wave DF (e.g. IGFBPs in D5 < D8 < D12-I), and that this process can be reversed by the injection of $PGF_{2\alpha}$ and the insertion of a progestin implant at d8 of the estrous cycle (Fig. 6-10; top-left, bottom-left&right). Furthermore, postranslational modifications of higher molecular weight forms of inhibin (196-116 kDa forms) into lower molecular weight forms (31 kDa) appears to be the other major operational mechanism taking place in the transitional process of atresia of DF. Collectively, the data indicates that although RIA concentrations of IGF-I and IGF-II remained unchanged across the four experimental groups studied, changes in the bioavailability of free IGF-I and IGF-II in follicular fluid may be decreased by increasing the amounts of low molecular weight IGFBPs throughout the transitional process of atresia of the first wave DF. Therefore, increased amounts of FF IGFBPs are likely to hasten the process of atresia by binding FF IGF-I and IGF-II and thereby negating their mitotic actions and stimulatory effects on steroidogenesis and inhibin production.

Four IGFBPs were detected in the FF of dominant and subordinate follicles (Fig. 6-7 and 6-8). In decreasing M_r, the four were: IGFBP-3 (49-43 kDa), IGFBP-2 (35 kDa), IGFBP-5 (30 kDa) and IGFBP-4 (28-22 kDa). Echternkamp *et al.* (1994), using immunoblot analysis detected IGFBP-2 (34 kDa), IGFBP-3 (44-40 kDa) and two additional bands: one at 29-27 kDa and the other at 22 kDa in bovine FF. It is very likely that the 27 and the 22 kDa bands reported by Echternkamp *et al.* (1994), correspond to the 28 and 22 kDa bands identified by immunoprecipitation as IGFBP-4. Furthermore, the 29 kDa band reported by Echternkamp *et al.* (1994) corresponds to the 30 kDa band reported in this study and identified by immunoprecipitation as IGFBP-5. Our results also agree with those reported by Stanko *et al.* (1994) that describe three IGFBPs (IGFBP-3 [43-39 kDa], IGFBP-2 [34 kDa] and IGFBP-5 [32-31 kDa]) in bovine FF by immunoblot analysis. In addition, Stanko *et al.,* (1994) reported one band at 24 kDa that was not identified but was hypothesized to be IGFBP-4. We report here a doublet at 28 and 22 kDa that is identified as IGFBP-4 in bovine FF. Even after long term exposure of the nitrocellulose membranes (8 d), we were not successful in identifying IGFBP-6 in bovine FF. Insulin-like growth factor-binding proteins of quite similar M_r have been described in FF of humans (Giudice, 1992; Zhou and Bondy, 1994), rats (Giudice, 1992; Liu *et al.,* 1993; Nakatani *et al.,* 1991) and pigs (Giudice, 1992; Grimes *et al.,* 1994). Therefore, using of ligand blot analyses and immunoprecipitations allowed identification of four distinct IGF-binding proteins, namely IGFBP-2, -3, -4 and -5 in bovine FF from dominant and subordinate follicles.

The absolute amount of total $_{LIGB}$IGFBPs were 61.4% higher in SF compared to DF (Fig. 6-8). Similar trends were observed between atretic and E_2 active DF. Day 12-I atretic DF had a 54.5% more total $_{LIGB}$IGFBPs compared to D5 E_2 active DF and 57.7%

more total $_{LIGB}$IGFBPs compared to D12+I E_2 active sustained DF (Fig. 6-8). Since, absolute amounts of IGFBP-3 were similar among experimental treatments and between dominant and subordinate follicles (Fig. 6-10, top-right), the increase in total $_{LIGB}$IGFBPs with atresia was due to an increase in the absolute amounts of IGFBP-2, -4 and -5 (Fig. 6-10, top-left, bottom-left&right). The absolute amounts of $_{LIGB}$IGFBP-2 were 100% higher in SF compared to DF; the absolute amounts of $_{LIGB}$IGFBP-4 and -5 were 218.6% and 217.0% higher in SF compared to DF. Furthermore, D12-I atretic DF had absolute amounts of $_{LIGB}$IGFBP-2, -4 and -5 that were 171.4%, 172.0% and 765.1% higher than D5 E_2 active DF, and were 83.7%, 211.4% and 175.7% higher than D12+I E_2 active-sustained DF (Fig. 6-10, top-left, bottom-left&right).

The observation that absolute amounts of $_{LIGB}$IGFBP-3 did not change among experimental treatments or between dominant and subordinate follicles is consistent with the reported absence of transcription and translation of IGFBP-3 mRNA in human (Zhou and Bondy, 1993), rat (Nakatani *et al.,* 1991) and porcine (Samaras *et al.,* 1992) ovarian follicles, and with the reported absence of IGFBP-3 in bovine granulosa and theca cell homogenates (Echternkamp *et al.,* 1994). Indeed, Echternkamp *et al.* (1994) speculated that the majority of IGFBP-3 measured in bovine follicular fluid originates as a sequestered pool from plasma or from extrafollicular tissue. The striking differences in the absolute amounts of IGFBP-2, -4 and -5 among dominant and subordinate follicles described in the present study agree with those reported in human (San Roman and Magoffin, 1993), rat (Liu *et al.,* 1993), pig (Grimes *et al.,* 1994) and bovine (Echternkamp *et al.,* 1994) ovarian follicles. However, instead of utilizing follicles during the follicular phase

(Echternkamp *et al.*, 1994; Grimes *et al.*, 1994; San Roman and Magoffin, 1993), the present study utilized first wave dominant and subordinate follicles obtained at very precise stages of development by means of an experimental model described in Chapter 3 to characterize FF profiles of these IGFBPs in E_2 active (D5), transitional E_2 active (D8), atretic (D12-I) and E_2 active sustained (D12+I) DF and cohort SF. This sequential sampling throughout the process of atresia (i.e. D5, D8 and D12-I) clearly shows an association of three IGFBPs with the process of atresia.

The mechanisms that regulate the amounts of IGFBP-2, -4 and -5 in FF of dominant and subordinate follicles are still not clear, but at least two mechanisms are thought to be operational (Liu *et al.*, 1993) and under FSH regulation. In the first mechanism, FSH may inhibit gene expression of IGFBP-2,- 4 and -5. Thus the FSH signal transduction mechanism acts at the gene level to either inhibit gene transcription of IGFBP-2, -4 and -5 or to increase degradation of IGFBP-2, -4 and -5 mRNAs (Liu *et al.*, 1993). *In vitro* studies clearly support this view (Adashi *et al.*, 1990, 1991; Liu *et al.*, 1993). Steady state levels of IGFBP-2 mRNA reported in this study also partially support this idea (Fig. 6-13). Although D5 and D8 DF had lower IGFBP-2 mRNA levels compared to D12-I and D12+I DF, no such differences were detected between D12+I and D12-I DF. Therefore our results support the idea of reduced gene transcription in E_2 active and in transitional E_2 active DF (D5 and D8) compared to D12-I atretic follicles but do not support this idea in E_2 active sustained DF where mRNA levels were similar to those for atretic DF (D12-I). In spite of high mRNA steady state levels for IGFBP-2 in D12+I DF, the lower absolute amounts of $_{\text{LIGB}}$IGFBP-2, -4 and -5 observed in these follicles compared to D12-I DF may indicate the presence of a postranslational mechanism such as degradation protease(s) to reduce IGFBPs in FF (Liu *et al.*, 1993).

In the second mechanism, FSH may stimulate the production of a protease(s) that cleaves IGFBP-2, -4 and -5 into lower molecular mass fragments which do not bind IGF in FF (Liu *et al.*, 1993). Several lines of evidence support this concept. *In vitro* studies done with rat granulosa cell cultures clearly show that FSH stimulates the production of proteases that degrade IGFBP-4 and -5 in culture medium (Liu *et al.*, 1993). Furthermore, there is evidence for specific protease activity in serum of pregnant rats (Davenport *et al.*, 1990, 1992) or porcine FF (Grimes and Hammond, 1994) which degrades IGFBP-3 into a fragment(s) that does not bind IGF-I and IGF-II with high affinity (Davenport *et al.*, 1990, 1992). Although plasma FSH concentrations decrease in response to inhibin produced by the DF (Findlay, 1993), FF FSH concentrations progressively accumulate in developing follicles in contrast to atretic follicles in which FSH concentrations remain at much lower levels (McNatty *et al.*, 1975). Therefore, higher concentration of FSH in FF would stimulate production of proteases that degrade IGFBP-4 and -5 in FF which would liberate more IGF to induce greater gene expression of P450 SCC (Magoffin *et al.*, 1990; Urban *et al.*, 1990), P450 ARO (Steinkamp *et al.*, 1988), induce greater stimulation of androgen biosynthesis (Hernandez *et al.*, 1988), aromatase activity (Adashi *et al.*, 1985a,b; Bergh *et al.*, 1991; Erickson *et al.*, 19989,1990), and inhibin production (Bicsak *et al.*, 1986). Such mechanisms would not be present in atretic DF and in SF because of low FSH concentrations in FF (McNatty *et al.,* 1975).

Another possible mechanism of IGFBPs acting at the ovarian level would be as general inhibitors of cell proliferation (Bicsak *et al.,* 1990). Support for this idea comes from *in vitro* studies with rat granulosa cell cultures (Bicsak *et al.,* 1990) where IGFBPs inhibited FSH stimulated [^{3}H] thymidine uptake and from having ruled out the actions IGFBPs at the FSH receptor (Bicsak *et al.,* 1990). Although demonstration of a direct interaction of IGFBP-2 or -3 with granulosa cells through cell surface receptors has not been described, the presence of the Arg-Gly-Asp motif in IGFBP-2 (Baxter and Martin, 1989), and the binding of IGFBP-1 to fibronectin and laminin (Jones *et al.,* 1993), gives further support to the idea that IGFBPs may function as general inhibitors of cell proliferation through direct interaction with granulosa cell receptors.

The relative proportion of the four different IGFBPs in FF were strikingly different between dominant and subordinate follicles (Fig. 6-11). In DF, IGFBP-3 represented 65.3% of the total $_{\mathrm{LIGB}}$IGFBPs whereas in SF IGFBP-3 represented only 42.5% of the total $_{\mathrm{LIGB}}$IGFBPs. Conversely, the relative proportion of IGFBP-2, -4 and -5 were 12.6%, 12.3% and 9.6% in DF and 17.5%, 23.2% and 16.7% in SF (Fig. 6-11). This decrease in the proportion of IGFBP-3 and increases in proportion of IGFBP-2, -4 and -5 in FF further support the concept of autocrine and paracrine mechanisms associated with the process of atresia of dominant and subordinate follicles. Insulin-like growth factor binding proteins-2, -4 and -5 are secreted by theca interna and granulosa cells whereas most IGFBP-3 present in FF originates from blood or from the extrafollicular tissue

(Echternkamp *et al.*, 1994). Therefore, presence of a higher proportion of IGFBP-2, -4 and -5 may reflect autocrine and paracrine mechanisms controlling atresia at the local level by reducing availability of IGF-I and IGF-II. The observation that IGFBPs levels were higher in FF of bovine follicles that contained oocytes with reduced developmental potential and in follicles with oocyte cumulus complexes that failed to develop to the blastocyst stage (Hazeleger *et al.*, 1993) further supports the involvement of IGFBPs in atresia of ovarian follicles and suggest that the amounts of IGFBPs in FF could be used as a potential marker for selection of follicles for aspiration to be used in *in vitro* maturation and fertilization schemes. Hence oocytes aspirated from follicles with high amounts of IGFBPs (either 2, 4 or 5) and low E_2 concentrations could be discarded whereas oocytes aspirated from follicles with low amounts of IGFBPs and high E_2 concentrations could be utilized in these schemes. The use of pumps to perform the follicular aspiration *in vivo* or use of individual syringes with abattoir ovaries would permit fast and specific collection of oocytes and FF. Although quantitation of IGFBPs in FF currently requires the use of ligand blots, the future potential of screening follicular fluid with commercial antibodies with enzyme immunoassay (EIA) may speed up processing of large quantities of FF samples. Similar tests are available for progesterone (Arnstadt and Cleere, 1981) and for early pregnancy factor determination (Threlfall, 1994) in plasma or milk samples.

Although IGFBP-2 mRNA steady state levels in unilateral ovariectomized cows were higher than in intact cows (Fig. 6-13, left), no increase in the absolute amounts of FF IGFBP-2 were detected (Fig. 6-10, top-left) in follicles collected from unilateral ovariectomized cows compared to follicles from intact cows. Therefore, proteases in FF (Liu *et al.*, 1993) could have reduced absolute amounts of IGFBPs to levels similar to those present in intact cows.

The changes in absolute amounts and relative proportions of $_{\mathrm{LIGB}}$IGFBP-2,- 4 and -5 during atresia of DF reported here represent an interesting example of potential intrafollicular autocrine and paracrine mechanisms to regulate follicular growth and development. Additional insights into this process were obtained by correlation, partial correlation and stepwise regression analyses (Table 6-2; Table 6-3; Fig. 6-14). These analyses confirmed two observations made by us in this study and by others (Echternkamp *et al.*, 1994). The first observation was the consistently high correlation observed among the low M_r IGFBPs (IGFBP-2, -4 and- 5) and the lack of correlation between IGFBP-3 with IGFBP-2, -4 or- 5. This observation gives further support to the previous view that bovine FF IGFBP-3 and low M_r IGFBPs have different sources (Echternkamp *et al.*, 1994). The majority of FF IGFBP-3 is likely to represent a pool derived from plasma or extrafollicular tissues. In contrast, the low M_r IGFBP-2, 4 and 5 are produced locally in the theca and/or granulosa cells (Echternkamp *et al.*, 1994). Furthermore, the SPC obtained from stepwise regression analysis clearly shows a high degree of association between the low M_r binding proteins, but no degree of association among these and IGFBP-3 (Fig. 6-14). The second observation was the high correlation between IGF-I and IGF-II concentrations and the $_{\mathrm{LIGB}}$IGFBP-3 present in FF (Table 6-2; Table 6-3). This suggests that perhaps IGF-I and IGF-II and IGFBP-3 all originate primarily from blood perfusion to the ovary. Similar results were obtained by stepwise regression analysis (Fig.

6-14), where high SPC were detected among the IGF-I and IGF-II and between IGF-I and IGFBP-3.

In summary, the following conclusions can be made from the present study: 1) the majority of subordinate follicles studied were atretic (PERT≥1), whereas the majority of D5, D8 and D12+I DF were E_2 active (PERT<1), 2) IGF-I and IGF-II concentrations in FF did not change during the process of atresia nor during a period of sustained dominance, 3) four distinct IGFBPs (IGFBP-2, -3, -4 and 5) were identified in bovine FF, and the total absolute amounts of these $_{LIGB}$IGFBPs were 61 % higher in SF compared to DF, 4) absolute amounts of IGFBP-3 were unchanged whereas the relative proportion was 22.5% lower in SF compared to DF, 5) the low M_r IGFBPs (-2, -4 and -5) dramatically increased with atresia of the DF and their relative proportion were increased in FF of SF, suggesting that these participate in autocrine and paracrine mechanisms that control atresia at the follicular level, and 6) levels of mRNA for IGFBP-2 did not parallel the changes in the absolute amounts or the relative proportions of IGFBP-2 in FF, suggesting that IGFBP-2 proteolysis in the FF may be one mechanism that the DF utilizes to diminish levels of low M_r IGFBPs and thereby increase the levels of bioactive IGF.

CHAPTER 7
GENERAL DISCUSSION AND CONCLUSIONS

Mammalian ovaries have a pool of primordial follicles, each consisting of an oocyte arrested in prophase I of meiosis and a single layer of undifferentiated squamous granulosa cells. In cattle, this pool develops during fetal life; follicles gradually and continually leave this resting pool to begin growth and development. Re-initiation of growth and development of the follicle is characterized morphologically by number of granulosa cell layers, extent of the thecal layer, antrum formation, size of the oocyte and signs of atresia (Erickson *et al.*, 1994; Fortune, 1994). The process of re-initiating growth of primordial follicles and subsequent folliculogenesis is a continual process such that follicles in the ovary form a hierarchy according to their functional status that culminates in ovulation or more likely atresia of the follicle (Fortune, 1994). This process of recruitment, selection and dominance is a highly selective process (Erickson *et al.*, 1994). Only a very small proportion ($\leq 0.1\%$) of the total follicles survive atresia and become dominant follicles (Aria, 1920). Hence, one of the most important questions in research on folliculogenesis concerns the mechanisms by which a cohort follicle is either selected to become a dominant ovulatory follicle or destined for programmed cell death (e.g. atresia; Erickson *et al.*, 1994).

Factors such as nutritional status and lactation, heat stress, follicular cysts, hormonal treatments (e.g. FSH, GH, bovine somatotropin [bST]) and various methods available for estrus synchronization affect the mechanisms regulating folliculogenesis and ultimately the animal's ability to ovulate a viable oocyte capable of being fertilized and supporting development of an offspring. Therefore, acquisition of additional knowledge in regulating mechanisms controlling folliculogenesis will permit improvement on: superovulatory responses combined with follicular aspiration for *in vitro* oocyte maturation, fertilization and blastocyst development; estrus synchronization methods; determination of times during follicular development that are sensitive to heat stress; nutritional management techniques to hasten ovarian activity in the postpartum dairy cow, and design efficacious treatments for follicular cysts.

The main objectives of this dissertation were to develop an experimental model to obtain first wave dominant and subordinate follicles at defined stages of development (Chapter 3), and to study endocrine and molecular aspects that regulate dominance and atresia of bovine ovarian follicles utilizing this model (Chapter 3, 4, 5 and 6). The second objective was focused on studying: changes in follicular fluid concentrations of P_4 and gene expression of P450 SCC and P450 3β-HSD (Chapter 3); changes in follicular fluid concentrations of A_4 E_2 and gene expression of P450 17α-HYD and P450 ARO (Chapter 4); changes in follicular fluid concentrations of $_{RIA}$INH, absolute amounts and proportions of $_{IMB}$INH, and gene expression of α- and β_A-inhibin (Chapter 5); and changes in follicular fluid concentrations of IGF-I and IGF-II, absolute amounts and proportions of $_{LIGB}$IGFBPs and gene expression of IGFBP-2 (Chapter 6). Furthermore, the standardized regression coefficients obtained from stepwise regression analyses represent a novel approach to study hormonal and molecular interrelationships in dominant follicles at different stages of dominance and atresia.

During the bovine estrous cycle, follicular growth is characterized by development of one or two dominant anovulatory follicles before the terminal preovulatory follicle is ovulated (Savio *et al.*, 1988, 1990; Sirois and Fortune, 1988; Ginther *et al.*, 1989abc). Therefore each of these waves has the potential to be used for studying follicular dominance and atresia in cattle. Sirois and Fortune (1990) have utilized a model where the luteal phase is extended with low levels of exogenous progesterone to promote development of the dominant follicle and hence study potential regulators of follicular dominance in cattle; whereas Voss and Fortune (1993a,b) utilized preovulatory follicles as an experimental model to study steroidogenic gene expression before and after the LH surge. Badinga *et al.* (1992) utilized the first wave dominant follicle as an experimental model to study endocrine and biochemical differences between d 5, 8 and 12 dominant and subordinate follicles whereas Savio *et al.* (1993) have used an experimental model to study sustained dominance in cattle in which turnover and atresia of the first wave dominant follicle can be prevented for a 15 d period by induction of luteolysis with $PGF_{2\alpha}$ and insertion of a progestin implant on d 8 of the estrous cycle. Combining these two approaches, we designed an experimental model that acquires follicles at known physiological stages compared to ovaries collected from the abattoir where follicles are at different and unknown stages of development. Cows were utilized during two complete experimental periods at the end of which the ovary bearing the dominant follicle was removed by unilateral ovariectomy. This permitted us to obtain an appreciable number of individual follicles (DF, n= 38 to 43; SF, n= 38 to 71) to be used repeatedly for different experimental measurements. In many of the responses studied, an experimental period effect was detected that reflected the effects of unilateral ovariectomy. The increase in gene expression of steroidogenic enzymes and of inhibin associated with the presence of only one ovary (period 2) was not associated with increased concentrations of steroids or increased amounts of inhibins in follicular fluid. An exception was an increase in FF P_4 and decreased in diameter of the subordinate follicle associated with presence of one ovary. These latter observations were associated with the presence of a less stronger dominant follicle after d 5 of the estrous cycle and hence a less atretic subordinate follicle which was translated in less FF P_4 and bigger diameter of these follicles at ovariectomy. However, we were not able to detect any interactions of experimental group or follicle type with experimental period and follicular dynamics described by ultrasonography agreed with earlier reports by Ginther *et al.* (1989), Savio *et al.* (1990), Badinga *et al.* (1992) and Lucy *et al.* (1992).

The main goal of this experimental model was to obtain follicles at defined stages during the transition from dominance to atresia of the first wave of follicle dominance and during a period of extended dominance created by an experimental induction in LH secretion to block the normal process of atresia. Indeed, we successfully obtained D5 E_2 active, D8 transitional E_2, D12-I atretic E_2 inactive and D12+I E_2 sustained dominant follicles as verified by the ratio of absolute concentrations of either FF E_2 to P_4 or P_4 to E_2, ultrasonographic data (size, day of wave, dominant or subordinate) and absolute FF

E_2 concentrations ($\leq$ or $>$ 50 ng/ml). Based upon any of these three thresholds, the majority of D5, D8 and D12+I dominant follicles were classified as E_2 active or non-atretic follicles. Conversely, D12-I dominant follicles and the majority of the subordinate follicles were classified as atretic or E_2 inactive. It was surprising that concentrations of FF P_4 combined with ultrasonographic data was not a good threshold to categorize atretic from E_2 active subordinate follicles. In summary, results from this study indicate that the ratio of absolute concentrations of FF E_2 to P_4 or the combination of FF E_2 and ultrasonographic data can be utilized to classify ovarian follicles in E_2 active (non-atretic) or E_2 inactive (atretic) when obtained at different days of the estrous cycle for experimental purposes.

Gene expression among the four steroidogenic enzymes studied were closely coupled in E_2 active (D5, D8 and D12+I) DF, but not in atretic DF (D12-I) or in the majority of the SF with the exception of SF from the second wave of follicle development (i.e. D12-I SF). This is supported by the high simple and partial correlations and standardized partial coefficients found between the mRNA levels of these four enzymes. Furthermore, gene expression decreased linearly throughout the transition of dominance to atresia of the first wave dominant follicle. However, concentrations of P_4, A_4 and E_2 in FF did not parallel gene expression. This uncoupling of gene expression with steroid production is very clear in at least three instances within this experimental model. The first uncoupling takes place in D8 transitional active DF (i.e., D5 vs D8), in which A_4 and E_2 FF concentrations decrease dramatically by 98% and 74% whereas gene expression for

P450 17α-HYD and P450 ARO decreased by only 25% and 20%. The second uncoupling takes place in the opposite direction (D12-I vs D12+I). P450 17α-HYD and P450 ARO gene expression in D12+I DF had increased by 28% and 95% while FF concentrations of A_4 and E_2 are increased by 1111% and 3521% compared to D12-I DF. The third uncoupling takes place in D12-I atretic DF where P450 SCC and P450 3β-HSD gene expression remains unchanged but FF P_4 concentrations are increased by 335%. Therefore, this uncoupling between gene expression of steroidogenic enzymes and corresponding changes in steroidal production as measured in FF could be explained via two different mechanisms. Perhaps subtle regulation of enzyme activity is determined either by postranslational modifications of enzymes or by cofactor availability (Hanukoglu, 1992). The other potential regulatory mechanism relates to steroidogenic enzyme mRNA stability and degradation and/or those factors that govern enzyme mRNA translation. These alternative mechanisms may alter steroid production in a manner unrelated to gene expression as measured by mRNA contents. Future studies should focus on this area to further identify the mechanism(s) responsible for the precipitous changes in FF steroids without the corresponding changes in gene expression.

The very distinct differences in plasma E_2 profiles observed between d 3 and d 5 of the estrous cycle for cows that underwent complete CL regression compared to those with incomplete CL regression has important implications. First, E_2 produced by the DF has an endocrine role at the uterine and pituitary level and potential endocrine, paracrine and autocrine role at the ovary level. The increased and more sustained concentration of plasma E_2 produced by active DF may have caused a longer priming period and facilitated luteolysis on d 8 of the estrous cycle in response to the injection of $PGF_{2\alpha}$. Conversely, cows with less estrogenic DF may have had an insufficient induction of endometrial oxytocin receptors since that the initial injection of $PGF_{2\alpha}$ was not able to induce a complete luteolytic mechanism. In addition it is now well documented that ovarian E_2 production potentiates the inhibition of FSH secretion caused by inhibin also produced by the dominant follicle (Findlay, 1993). The endocrine, paracrine and autocrine effects of FF E_2 produced by the DF have been suggested to be mediated through inhibition of P450 17α-HYD gene expression and enzyme activity (Magoffin and Erickson, 1982). Hence, at the endocrine level, high concentrations of FF E_2 found in DF could selectively inhibit enzyme activity in the cohort of SF of both ovaries. This view is supported partially by the decrease in gene expression for P450 17α-HYD (long term effects; Magoffin and Erickson, 1982) in D5 SF and fully supported by the dramatic reduction in FF A_4 (competitive inhibition; Magoffin and Erickson, 1982) observed in SF. The autocrine effects of high FF E_2 present in D5 DF also could be manifested through product inhibition and decreased gene expression in D8 DF. In the present study, a sharp decrease in FF A_4 concentrations and a minor decrease in P450 17α-HYD gene expression in D8 DF supports this concept. Due to the high correlation between FF and concentrations of plasma E_2, and the high accuracy utilizing of FF E_2 concentrations ($\leq$ or $>$ 50 ng/ml) as a threshold to classify follicles as E_2 active or atretic, it is tempting to propose FF E_2 concentrations as a marker of follicular health for aspirated follicles whose oocytes are used for *in vitro* maturation and fertilization schemes.

The FF concentrations of $_{RIA}$INH, absolute and relative proportions of $_{IMB}$INH, and α- and β_A-inhibin mRNA steady state levels were characterized during dominance and atresia of first wave dominant and subordinate follicles. Qualitative immunoblot analyses of non-reduced FF identified eight inhibin forms with apparent molecular mass of >300, 196-116, 80, 58, 49, 28 and 25 kDa. Similar results were reported by Ireland and Ireland (1994b). Furthermore, Good *et al.* (1994), using an antibody against the β-subunit further identified the 49 and the 29 kDa bands as monomeric forms while the other forms are dimeric forms of inhibin (α- β_A). With this classification, 75% of the $_{IMB}$INHs present in FF are dimeric and 25 % are monomeric in agreement with results reported by Ireland and Ireland (1994b).

The total absolute amounts of $_{IMB}$INH measured in FF decreased with atresia of the DF; however the individual absolute amounts and proportion of different $_{IMB}$INH forms present in FF showed two distinct patterns of change during the process of atresia. The 196-116 kDa form was identified as the predominant form in E_2 active DF representing approximately 50% of the total forms present in FF. Conversely, the 31 kDa form was the primary $_{IMB}$INH associated with atresia of the DF, increasing from 3% to 15% in D12-I atretic follicles. A modest increase in the 300 kDa forms also was detected with atresia. In sharp contrast to these dynamic changes of the different $_{IMB}$INH, $_{RIA}$INH concentrations in FF remained fairly constant across all experimental groups and only differences between DF and SF were detected. This findings support the emerging concept that $_{RIA}$INH concentrations do not reflect the dynamic changes of the different forms of inhibin in FF that are detected by immunoblot analysis and associated with atresia. Expression of α- β_A-

inhibin mRNA was elevated during the first 8d of the estrous cycle but then decreased 35 to 70% with atresia of the DF. Furthermore, the hormonal changes induced in the D12+I group leading to sustained dominance maintained absolute amounts of $_{IMB}$INH forms similar to those of D5 or D8 DF but without a change in gene expression. A similar example, but in opposite direction happens with the increase in the 31 kDa form $_{IMB}$INH with no concomitant increase in gene expression. Therefore, changes in contents of $_{IMB}$INH contents without the corresponding changes in gene expression represent striking examples of intrafollicular processing of inhibin molecules from higher to lower molecular weight forms during atresia of the DF.

The use of stepwise regression analysis helped to gain additional information as to the potential processing routes among inhibin molecules throughout the stages of DF function (Chapter 5, Fig. 5-14). Two processing routes were identified with atresia of the DF. The first processing route comprises the stepwise reduction of 196-116 kDa to 80 kDa to the 31 kDa form, and this is confirmed by the strong negative association between the 196-116 and the 31 kDa forms in FF. The second route involves a single step from 196-116 kDa to the 300 kDa form and its association with atresia is confirmed by the increased amounts present in D12-I atretic DF. Furthermore, the $_{IMB}$INH profiles in FF indicate that the 196-116 kDa form is the most likely candidate for exerting a negative feedback on FSH secretion from the pituitary. The 49 kDa form may be involved as an endocrine modulator of FSH binding to its receptor and subsequent bioactivity. Schneyer *et al.* (1991) demonstrated that a form of inhibin or the free α-subunits of inhibin precursors may inhibit FSH action by competing for the FSH receptor. The observation that the 49 kDa monomeric form of inhibin is high in D5 and D8 DF indicate this molecule may be a potential competitive binder to FSH receptors and block recruitment of new follicles.

The FF concentrations of IGF-I and IGF-II during dominance and atresia of first wave dominant and subordinate follicles also was studied. Therefore, an IGF separation procedure using acidification and C_{18} Sep-Pak chromatography was validated for bovine follicular fluid by demonstrating complete removal of IGFBPs and a parallelism of isolated material with displacement curves in representative RIAs. Results from both RIAs revealed that concentrations of both growth factors remained very much unchanged between experimental groups and follicles. However, very dramatic changes in amounts and proportions of FF IGFBPs were detected. Ligand blots and immunoprecipitation analyses identified at least four IGFBPs in bovine FF: IGFBP-3 (49 and 43 kDa), IGFBP-2 (35 kDa), IGFBP-5 (30 kDa) and IGFBP-4 (28 and 22 kDa). Similar IGFBPs were detected by Echternkamp *et al.* (1994) and Stanko *et al.* (1994) using ligand blot analysis; however, only three IGFBPs have been identified by immunoprecipitation in bovine FF (IGFBP-2, 3 and -5; Stanko *et al.*, 1994). This is the first study demonstrating the presence of IGFBP-4 in bovine FF. Subordinate follicles in all experimental groups had higher total absolute amounts of $_{LIGB}$IGFBPs (61%) and absolute amounts of IGFBP-2 (100%), IGFBP-4 (200%) and IGFBP-5 (217%) compared to DF. The D12-I atretic DF had higher levels of IGFBP-2, -4 and -5 compared to either D5 E_2 active or D12+I E_2 DF. However, IGFBP-3 did not change among experimental treatments or between dominant and subordinate follicles. Similar results have been reported in human (San

Roman and Magoffin, 1993), rat (Liu *et al.*, 1993), pig (Grimes *et al.*, 1994) and bovine (Echternkamp *et al.*, 1994) ovarian follicles; however, the results from the present study were determined in dominant and subordinate follicles at very precise stages of development. Collectively, these results support the concept that FF IGFBPs may selectively bind IGF-I present in FF and therefore reduce its potential trophic activity (Ui *et al.*, 1989) and the well documented effects of IGF-I on granulosa cell expression of steroidogenic enzymes (Magoffin *et al.*, 1990; Steinkamp *et al.*, 1988), androgen biosynthesis (Hernandez *et al.*, 1988), aromatase activity (Adashi *et al.*, 1985) and inhibin production (Bicsak *et al.*, 1986). The stimulatory effects of IGF-I on granulosa cells of D12-I atretic DF and SF were likely reduced and this view is supported by the very low concentrations of A_4 and E_2 in FF and lower steady state levels of P450 17α-HYD and P450 ARO in these follicles.

The mechanisms that take place in E_2 active DF to maintain very low absolute amounts of $_{\mathrm{LIGB}}$IGFBPs have not been elucidated; however two FSH dependent mechanisms are postulated. The FSH signal transduction mechanism may act at the gene level to either inhibit gene transcription of the low M_r IGFBPs or to increase degradation of the low M_r IGFBPs (Liu *et al.*, 1993). Alternatively, FSH may stimulate production of protease(s) that cleaves the low M_r IGFBPs into lower molecular mass fragments which do not bind to FF IGF and thus are not visualized in ligand blots (Liu *et al.*, 1993). The first mechanism is partially supported in the present study by the lower steady state levels of IGFBP-2 present in D5 and D8 DF compared to D12-I DF whereas the second mechanisms is supported, although not directly tested, by the striking reduction in the absolute amounts of low M_r IGFBPs measured in FF of E_2 active DF. In the present study, IGFBPs in FF were quantitated by ligand blot analysis. Therefore, if FF proteases induced by FSH degraded low M_r IGFBPs in FF, then the resulting fragments would not bind the ^{125}I-IGF-II and would not be detected. Future studies should address what mechanisms are responsible for the reduction in low M_r IGFBPs in E_2 active DF as described in this study.

Table 7-1 depicts a summary of the most important simple correlations found between all the variables studied. Each dependent variable studied is shown in the left column and the six highest significant coefficient are shown to the right in decreasing order. This table combines results depicted in Tables 4-2, 5-2, and 6-2. Collectively, data support the involvement of E_2, A_4, IGF-I, and the 196-116 kDa and the 49 kDa forms of $_{IMB}$INH in the process of dominance as demonstrated by high correlation coefficients observed among these variables. In addition, P_4, IGFBP-2, -4, -5, and the 31 kDa form of $_{IMB}$INH in FF are involved in the process of atresia by the high correlations coefficients observed within each group but the lack of correlation with the former group.

The present results and those of others (Erickson *et al.*, 1994; Findlay, 1993; Fortune, 1994; Hillier, 1994; Magoffin and Erickson, 1994) lead to the following theoretical models depicting potential mechanisms for dominance (Figure 7-1) and atresia (Figure 7-2) of bovine ovarian follicles. From a pool of recruited follicles, one follicle is selected according to a precise developmental program that involves a defined sequence or temporal pattern of gene expression. The action of LH initiates gene expression of

Table 7-1. Summary of the six highest simple correlation coefficients found for each dependent variable studied.

Dependent variable	1st Indep. Variable	2nd Indep. Variable	3rd Indep. Variable	4th Indep. Variable	5th Indep. Variable	6th Indep. Variable
P_4	IGFBP-2 0.45 (<0.01)	31 kDa Inh 0.40 (<0.01)	IGFBP-5 0.32 (0.03)	IGFBP-2 0.29 (0.05)	SCC 0.34 (0.02)	3 β-HSD 0.32 (0.03)
SSC mRNA	ARO 0.99 (<0.01)	α-bInhibin 0.98 (<0.01)	3 β-HSD 0.97 (<0.01)	17 α-HYD 0.96 (0.01)	β_A-bInhibin 0.90 (<0.01)	BP2mRNA 0.89 (<0.01)
3 β-HSD mRNA	ARO 0.97 (<0.01)	SSC 0.97 (<0.01)	α-bInhibin 0.97 (<0.01)	17 α-HYD 0.96 (<0.01)	BP-2mRNA 0.89 (<0.01)	β_A-bInhibin 0.97 (<0.01)
A_4	E_2 0.74 (<0.01)	$_{\mathrm{RIA}}$bINH 0.48 (<0.01)	IGF-II 0.38 (0.01)	IGF-I 0.33 (0.03)	49 kDa Inh 0.31 (0.04)	
17 α-HYD mRNA	ARO 0.98 (<0.01)	α-bInhibin 0.97 (<0.01)	SSC 0.96 (<0.01)	3 β-HSD 0.96 (<0.01)	β_A-bInhibin 0.94 (<0.01)	BP2mRNA 0.94 (<0.01)
E_2	$_{\mathrm{RIA}}$bINH 0.77 (0.01)	A_4 0.74 (<0.01)	196-116 0.67 (<0.01)	49 kDa 0.64 (<0.01)	80 kDa 0.62 (<0.01)	IGF-I 0.57 (<0.01)
ARO mRNA	SSC 0.99 (<0.01)	17 α-HYD 0.98 (<0.01)	3 β-HSD 0.98 (<0.01)	α-bInhibin 0.98 (<0.01)	β_A-bInhibin 0.91 (<0.01)	BP2mRNA 0.91 (<0.01)

Table 7-1. (continued).

Dependent variable	1st Indep. Variable	2nd Indep. Variable	3rd Indep. Variable	4th Indep. Variable	5th Indep. Variable	6th Indep. Variable
$_{\text{RIA}}$bINH	E_2 0.77 (<0.01)	196-116 0.75 (<0.01)	80 kDa 0.75 (<0.01)	49 kDa 0.70 (<0.01)	300 kDa 0.68 (<0.01)	IGF-I 0.66 (<0.01)
$_{\text{IMB}}$bINH 300 kDa	196-116 0.95 (<0.01)	80 kDa 0.94 (<0.01)	49 kDa 0.89 (<0.01)	28 kDa 0.78 (<0.01)	$_{\text{RIA}}$bINH 0.68 (<0.01)	E_2 0.60 (<0.01)
$_{\text{IMB}}$bINH 196-116	300 kDa 0.95 (<0.01)	80 kDa 0.95 (<0.01)	49 kDa 0.91 (0.01)	28 kDa 0.81 (<0.01)	$_{\text{RIA}}$bINH 0.75 (<0.01)	E_2 0.67 (<0.01)
$_{\text{IMB}}$bINH 80 kDa	196-116 0.95 (<0.01)	300 kDa 0.94 (<0.01)	49 kDa 0.89 (0.01)	28 0.75 (<0.01)	$_{\text{RIA}}$bINH 0.75 (<0.01)	E_2 0.64 (<0.01)
$_{\text{IMB}}$bINH 49 kDa	196-116 0.91 (<0.01)	300 kDa 0.89 (<0.01)	80 kDa 0.89 (<0.01)	28 kDa 0.89 (<0.01)	$_{\text{RIA}}$bINH 0.70 (0.01)	E_2 0.64 (<0.01)
$_{\text{IMB}}$bINH 31 kDa	28 kDa 0.55 (<0.01)	80 kDa 0.43 (<0.01)	P_4 0.40 (<0.01)	300 kDa 0.40 (<0.01)	$_{\text{RIA}}$bINH 0.32 (<0.01)	IGFBP-3 0.32 (0.03)
$_{\text{IMB}}$bINH 28 kDa	196-116 0.81 (<0.01)	49 kDa 0.89 (<0.01)	300 kDa 0.78 (<0.01)	$_{\text{RIA}}$bINH 0.59 (<0.01)	31 kDa 0.55 (<0.01)	IGFBP-3 0.55 (<0.01)
α-bInhibin mRNA	SSC 0.98 (<0.01)	ARO 0.98 (<0.01)	3 β-HSD 0.97 (0.01)	17 α-HYD 0.97 (<0.01)	β_A-bInhibin 0.89 (<0.01)	BP-2mRNA 0.89 (<0.01)
β_A-bInhibin mRNA	17 α-HYD 0.92 (<0.01)	ARO 0.91 (<0.01)	SCC 0.90 (<0.01)	3 β-HSD 0.89 (<0.01)	α-bInhibin 0.89 (<0.01)	BP-2mRNA 0.89 (<0.01)

Table 7-1. (Continued).

Dependent variable	1st Indep. Variable	2nd Indep. Variable	3rd Indep. Variable	4th Indep. Variable	5th Indep. Variable	6th Indep. Variable
IGF-I	IGF-II 0.87 (<0.01)	E_2 0.57 (<0.01)	${}_{RIA}$bINH 0.59 (<0.01)	17 α-HYD 0.43 (<0.01)	β_A-bInhibin 0.42 (<0.01)	BP-2mRNA 0.42 (<0.01)
IGF-II	IGF-I 0.87 (<0.01)	E_2 0.61 (<0.01)	IGFBP-3 0.62 (<0.01)	${}_{RIA}$bINH 0.66 (<0.01)	49 kDa 0.46 (<0.01)	β_A-bInhibin 0.44 (<0.01)
IGFBP-3	IGF-I 0.64 (<0.01)	IGF-II 0.62 (<0.01)	28 kDa 0.55 (0.01)	196-116 0.37 (0.01)	β_A-bInhibin 0.37 (0.01)	α-bInhibin 0.36 (0.02)
IGFBP-2	IGFBP-4 0.87 (<0.01)	IGFBP-5 0.78 (<0.01)	IGF-I 0.39 (<0.01)	IGF-II 0.31 (0.03)	IGFBP-3 0.33 (0.02)	P_4 0.29 (0.05)
IGFBP-4	IGFBP-5 0.95 (<0.01)	IGFBP-2 0.87 (<0.01)	P_4 0.45 (<0.02)			
IGFBP-5	IGFBP-4 0.95 (<0.01)	IGFBP-2 0.78 (<0.01)	P_4 0.32 (<0.01)			
IGFBP-2 mRNA	17 α-HYD 0.94 (<0.01)	ARO 0.91 (<0.01)	α-bInhibin 0.91 (<0.01)	β_A-bInhibin 0.89 (<0.01)	SCC 0.89 (<0.01)	3 β-HSD 0.89 (<0.01)

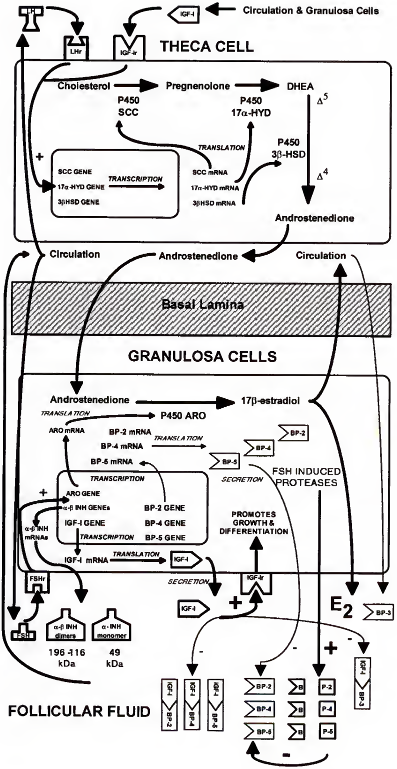

Figure 7-1. Diagram of a theoretical model collating potential mechanisms involved in dominance of bovine ovarian follicles.

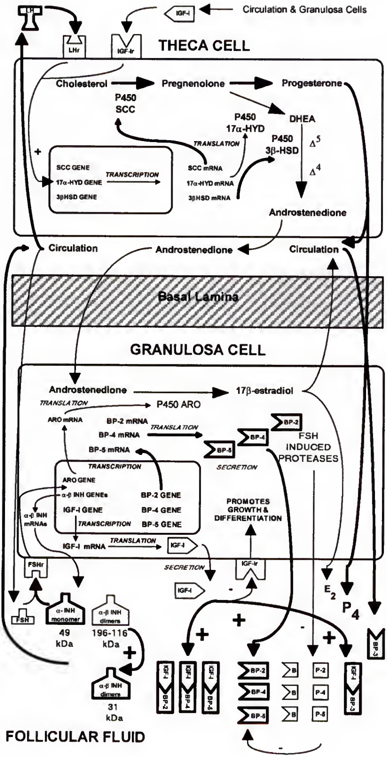

Figure 7-2. Diagram of a theoretical model associated with potential mechanisms regulating atresia of bovine ovarian follicles.

P450 SCC, P450 3β-HSD and P450 17α-HYD to ensure timely production of A_4 through the Δ^5 pathway in theca cells. Furthermore, granulosa cells under FSH stimulation initiate P450 ARO gene expression ultimately leading to an aromatization of thecal androgens to 17β-estradiol. Follicular fluid E_2 stimulates IGF-I production by granulosa cells and of A_4 production in theca interstitial cells (Hammond *et al.*, 1985). In addition, inhibins and E_2 reach the pituitary via the peripheral circulation and inhibit FSH secretion (Findlay, 1993), and E_2 alone reaches the other follicles in ipsilateral and contralateral ovaries and inhibit A_4 production in subordinate follicles (Magoffin and Erickson, 1982). Locally produced IGF-I and E_2 potentiate in an autocrine fashion the FSH stimulated E_2 and inhibin production in granulosa cells and also potentiate in a paracrine fashion the LH stimulated A_4 production in theca cells (Findlay, 1993). In addition, IGF-I promotes growth and differentiation in granulosa and theca cells (Erickson *et al.*, 1994; Magoffin and Erickson, 1994). The FSH stimulation of granulosa cells is likely to either inhibit gene transcription and/or translation of IGFBP-2, -4 and -5 or increase mRNA degradation of IGFBP-2, -4 and -5 in these cells (Liu *et al.*, 1993). Available evidence also suggests that FSH may also stimulate production of protease(s) that cleave FF IGFBP-2, -4 and -5 into low molecular mass fragments that do not bind to FF IGF and therefore reduce absolute amounts and proportions of these binding proteins in DF when compared to atretic DF and SF (Liu *et al.*, 1993). The increased production of E_2 and inhibin from the DF decreases FSH secretion and hence restricts growth of SF. However, the DF survives this decrease in FSH stimulation by amplifying effects of IGF-I, which sensitizes cells of the DF to gonadotropin stimulation. Conversely, the cohort of SF lacks these amplifying mechanisms due to the presence of low molecular weight IGFBP-2, -4 and -5 which reduces availability of FF IGF-I and therefore exacerbates gonadotropin deprivation and follicles become atretic. The 196-116 kDa dimeric form of inhibin is present at high concentrations in FF of D5 and D8 DF and is a most likely candidate to inhibit FSH secretion. The 49 kDa monomeric form present at d5 and d8 in FF of DF is hypothesized to act in an endocrine manner back on ovarian follicular populations to compete with the FSH receptor. This would be analogous to the inhibition of FSH binding by free α-subunit precursors to granulosa cells *in vitro* (Schneyer *et al.*, 1991). Such a competition with FSH for the FSH receptor and would reduce available FSH to granulosa cells of subordinate follicles and block recruitment or growth.

A theoretical model that encapsulates potential mechanisms involved in regulating atresia of subordinate follicles is depicted in Figure 7-2. Subordinate follicles (5 to 10 mm) have fewer gonadotropin receptors and therefore their responsiveness to gonadotropins is reduced (Ireland and Roche, 1983). This is further amplified by the reduction in FSH secretion due to inhibin and E_2 produced by the dominant follicle. Whereas the 196-116 kDa dimeric form of inhibin may inhibit FSH secretion, the 49 kDa monomeric form is a likely candidate to competes in the subordinate follicles with FSH for binding to the FSH receptor. The lower FSH exposure (Adams *et al.*, 1992) and the lower FSH FF concentration present in SF (McNatty *et al.*, 1975) are not sufficient to suppress the mechanisms that DF have available to keep FF IGFBP concentrations at low levels (Liu *et al.*, 1993). Hence, high amounts of IGFBP-2, -4 and -5 are present in FF of SF and limit the bioavailability of IGF-I to granulosa cells (locally produced and from peripheral circulation). A sequestration of IGF-I by the IGFBPs deprives granulosa and theca cells of potentiating effects IGF have on gonadotropin stimulated steroidogenesis of granulosa and theca cells. Furthermore, the reduction in free FF IGF-I in SF deprives new follicles of the growing promoting activity and mitotic activity that otherwise allowsgranulosa and theca cells to proliferate and differentiate. Thus, recruitment of new follicles (2 to 4 mm) is inhibited through this mechanism.

These mechanism regulating function of SF also are applicable to atretic DF. However there are at least three potential mechanisms regulating the transition from dominance to atresia of the first wave dominant follicle. The first mechanism is the reduction in LH pulse frequency due to increased plasma P_4 production from the newly formed CL (Savio *et al.*, 1993a). This decrease in LH support (Savio *et al.*, 1993a) is concomitant with the sharp drop in steroidogenesis around d8 of the estrous cycle (Fig. 4-2), and LH stimulates P450 17α-HYD gene expression (Erickson *et al.*, 1985). Thus, this decrease in LH support is partially responsible of the sharp drop in steroidogenesis at d8 of the estrous cycle. Product inhibition of P450 17α-HYD enzyme activity by high concentrations of FF E_2, is thought to be the second mechanism responsible for initiation of the process of atresia of the first wave dominant follicle (Magoffin and Erickson, 1982). In addition, postranslational processing of the 196-116 kDa dimeric forms of inhibin into the lower molecular weight 31 kDa dimeric form of inhibin sharply increases with atresia of the DF (Chapter 5, Fig. 5-6) supports the involvement of this molecule as a third potential mechanism to induce atresia of the DF. However, further research needs to be conducted in this area to determine the role of this molecule to induce atresia of the DF.

Pregnancy rate of a dairy herd is a product of heat detection rate and conception rate of cows inseminated at detected estrus. Under current production practices, cow numbers and/or milk production per cow of the herd has increased to decrease fixed costs and maintain profitability of the dairy operation. Increased milk production and reduced fertility have re-enforced the need to optimize reproductive management practices to maximize profits, and this is a tremendous challenge for dairy farmers and veterinarians. Follicular dynamics during the postpartum period and estrous cycle are altered markedly by metabolic demands of lactation (Lucy *et al.*, 1991). Similar changes in follicular dynamics lead to development of persistent follicles in the normal estrous cycles and during estrus synchronization programs (Savio *et al.*, 1993a). The present dissertation was focused to study some of the basic endocrine and molecular mechanisms controlling dominance and atresia of bovine follicles. Such knowledge will improve present superovulatory and estrus synchronization schemes, nutritional management techniques that hasten ovarian activity in the early postpartum dairy cow, and development of new treatments for ovarian follicular cysts in cattle.

For instance, superovulatory treatments are initiated at approximately d 10.5 of estrous cycles by daily administration of FSH at 12 h intervals during 4 days to induce a new wave of follicle recruitment. This treatment coincides with the initiation of the second follicular wave in cows experiencing three follicular waves but would start during the transition of a first wave dominant follicle from E_2 active to atretic in cows with two waves of follicular development. In the present study, half of the follicles less than 10 mm in diameter at d 12 of the estrous cycle were atretic (D12-I experimental group) based upon the EPR, the presence of low M_r IGFBPs (-2, -4 and -5) and 31 kDa forms of inhibin. Conversely, the remaining follicles were E_2 active based upon the EPR, and the reduced amounts of low M_r IGFBPs (-2, -4 and -5) and 31 kDa form of inhibin. Starting a superovulatory regime at d 10.5 of the estrous cycle has the potential to provide enough FSH to "rescue" follicles from atresia and hence include them in the pool of newly recruited follicles. Whereas newly recruited follicles would have an hormonal milieu comparable to a D5 E_2 active DF, rescued atretic follicles will have an hormonal milieu comparable to D12-I atretic DF. The lower EPR, higher amounts of low M_r IGFBPs, and 31 kDa form of inhibin in atretic follicles "rescued" with the superovulation regime may not allow subsequent normal development of the follicle and oocyte before and after ovulation. This could explain the high degree of variability in embryo recovery and quality obtained from these programs. As an alternative, we propose the use of follicular aspiration *in vivo* or *in vitro* and screening of FF for E_2 and/or one M_r IGFBP could be used to discard the potentially atretic follicles before they are utilized further in systems involving *in vitro* maturation and *in vitro* fertilization.

Fertility of cows that have ovulated a persistent follicle is reduced by 25% (Savio *et al.*, 1993b; Wehrman *et al.*, 1993). This reduction appears to be due to ovulation of an oocyte with asynchronous nuclear and cytoplasmic maturation (Muhm *et al.*, 1994). In the present study, a persistent follicle was induced for a 4d period. Although the intrafollicular characteristics regarding to changes in IGFBPs, FF forms of inhibins and steroids were similar to those detected in healthy follicles at D5. Hence alterations in the process of oocyte maturation leading to poor fertility may be associated with prolonged maintenance for a period beyond 4 days. For example, a sustained period of low peripheral P_4 concentrations in sustained E_2 may lead to a persistent pattern of elevated basal secretion of LH that stimulates premature maturation of the oocyte. When a persistent follicle is sustained for a period of 10 d, plasma E_2 concentrations are increased by 600% (Savio *et al.*, 1993a).

An experimental model was designed to conduct a detailed study involving three endocrine and molecular aspects that are thought to play an important role in the process of dominance and atresia in first wave individual follicles in cattle. Changes in FF steroids concentrations (P_4, A_4, E_2) were several orders of magnitude higher that rates of change in gene expression of P450 SCC, P450 3β-HSD, P450 17α-HYD, P450 ARO, suggesting that regulation of steroidogenic enzyme activity and/or mechanisms that regulate enzyme translation or stability may play a more important role in regulating steroidal production of transitional estrogen active dominant follicles. Inhibin in follicular fluid appears to undergo extensive postranslational processing during of first wave dominant follicles from functional dominance to atresia. Two inhibin molecules were identified as potential candidates for exerting regulatory effects at the pituitary and ovarian level. The 196-116 kDa dimeric may act on the pituitary to inhibit FSH secretion whereas the 49 kDa monomeric form is proposed to compete with FSH for its own receptor in subordinate follicles and in new recruitable follicles. Although increases in the amounts of the 31 kDa dimeric form of inhibin were associated with atresia, the role of this molecule is not clear. A very distinct pattern of low molecular weight IGFBPs was described in FF of atretic dominant follicles and the majority of subordinate follicles studied (except newly recruited follicles in D12-I). These increases in the amount and in the proportions of IGFBPs (-2, -4, -5) in FF of atretic follicles is thought to be responsible for limiting IGF-I stimulation. This would negate the growth promoting activity, mitotic activity and amplification of gonadotropin induced steroidogenesis that is normally present in estrogen active dominant follicles. A novel approach for studying follicular interrelationships was utilized to gain additional knowledge leading to the development of two theoretical models accounting for the development of dominance (Fig. 7-1) and atresia (Fig. 7-2) of first wave dominant follicles in cattle.

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APPENDIX 1
RNA EXTRACTION IN OVARIAN FOLLICLE WALLS

GENERAL

The most important consideration in the preparation of RNA is to rapidly and efficiently inhibit the endogenous ribonucleases which are present in virtually all living cells. Another important concern in the preparation of RNA is to avoid accidental introduction of trace amounts of ribonucleases from hands, glassware and solutions. It is therefore imperative to

1. use gloves throughout all procedures.
2. autoclave reagents that need to be sterile.
3. use only sterile tubes, pipettes, tips and glassware.

Ovarian dominant follicles weight between 400 to 1000 mg and subordinate follicles between 50 to 400 mg. The protocol has been scaled down to 500 mg of tissue sample.

PROTOCOL

DONE

___ 1. Label 50 ml conical centrifuge tubes and let sit on ice.
___ 2. Add 5 ml of guanidium thiocyanate to each tube,
___ 3. Add 500 μl of 2 M sodium acetate (pH 5.0) to each tube and mix gently.
___ 4. Add 500 mg of fresh or frozen tissue (cut into pieces) to each tube.
___ 5. Add 39.0 μl of 2-mercaptoethanol to each tube.
___ 6. Homogenize tissue with a polytron tissue homogenizer for three 15 sec bursts. Keep tissue on ice during homogenization.
___ 7. Add 5 ml of water-saturated phenol and 1 ml of chloroform. Cap and mix by shaking. Change to 15 ml conical centrifuge tubes.
___ 8. Centrifuge at 4000 rpm for 15 min.
___ 9. Transfer upper phase to a fresh 15 ml tube. Be careful not to take any of the bottom layer.
___ 10. Add 5 ml of isopropanol to precipitate RNA. Mix gently and place in a -80 °C freezer for 1 h.
___ 11. Centrifuge at 4000 rpm for 10 min and discard the supernatant.

__ 12. Resuspend the pellet in 1 ml of 4 M LiCl. Dislodge RNA pellet from bottom of the tube using a sterile transfer pipette. RNA does not go into solution.

__ 13. Centrifuge at 4000 rpm for 10-15 min to repellet.

__ 14. Pour off the supernatant and blot on a clear paper towel.

__ 15. Resuspend the pellet in 1 ml of RNA buffer (10 mM Tris, 1 mM EDTA, 0.5% SDS, pH 7.5) and let sit at RT until RNA goes into solution.

__ 16. Transfer content of the tube into a 15 ml conical tube.

__ 17. Add 1 ml of chloroform and vortex for 15 sec.

__ 18. Centrifuge at 4000 rpm for 10 min.

__ 19. Collect upper phase with sterile pipette and transfer into a fresh 15 ml conical tube.

__ 20. Add 100 μl of 2 M sodium acetate (pH 5.0) and mix gently.

__ 21. Add 1 ml of isopropanol and mix.

__ 22. Store overnight in a -80 °C freezer.

__ 23. Centrifuge at 4000 rpm for 30 min. Insoluble pellet should contain pure RNA after centrifugation.

__ 24. Pour off supernatant, blot and add approximately 500 μl of 70% ethanol to wash pellet. Recentrifuge at 4000 rpm for 10 min. Discard supernatant and blot on adsorbent paper. Dry 12-15 min.

__ 25. Resuspend in 100 μl sterile DDH$_2$O. Let sit at RT for 10 min, vortex gently and transfer to 1.6 ml bullet tubes.

__ 26. Pipet 10 μl of sample to a 12 X 75 test tube.

__ 27. Add 990 μl sterile water to 10 μl sample and read at 260:280 nm.
To calculate ug RNA use the following equation:
O.D. 260 (_) x dilution (_) x 33 μg/ml x tot. sample vol = __ Tot. μg RNA.

To determine RNA purity: calculate 260:280 ratio.
a. Ratios between 1.8 - 2.0 are good.
b. Ratios less than 1.2 may indicate substantial protein contamination.

REAGENTS AND SOLUTIONS

- Guanidine Thiocyanate:
  236.31 g Guanidine Thiocyanate (GT; 4M; Fisher Scientific BP221-1)
  3.67 g Sodium Acetate (25mM; Fisher Scientific BP327-1)
  2.50 g Sarcosyl ((Fisher Scientific BP234-500)
  Complete up to 500 ml volume with DDH$_2$O
  GT is light sensitive (cover with foil or use a brown bottle), adjust pH 7.0. Autoclave for 30 minutes at liquid setting. Store in refrigerator.
  2-Mercaptoethanol (0.1 M ; Sigma M-3148). Add 7.8 μl/ml **just prior** to adding the sample

- Lithium Chloride:
  16.95 g Lithium Chloride (4M; Fisher Scientific L121-100) in 100 ml DDH$_2$O
  Autoclave

- Sodium Acetate:
  68.04 g Sodium Acetate (2M; Fisher Scientific BP334-500) in 250 mm DDH$_2$O (Add only 100 ml of DDH$_2$O and pH to 5.0 with Acetic Acid (Fisher Scientific A38^{S}-212)
  Autoclave

- Water Saturated Phenol:
  Melt phenol (Fisher Scientific BP226-500) at 56°C. Add equal volume of DDH$_2$O. Keep in refrigerator/freezer (warm to 37°C). Phenol will be on bottom phase

- RNA Buffer (10 mM Tris, 1mM EDTA, 0.5% SDS, pH 7.5):
  10 mM Tris = 1 ml stock (Stock 1M = 60.57 g Tris [Fisher Scientific BP152-1000] + 500 ml DDH$_2$O; pH 7.5)
  1 mM EDTA = 200 μl stock (Stock 0.5M = 93.06 g EDTA [Fisher Scientific BP120-1000] + 500 ml DDH$_2$O; pH 8.0 [adjust pH periodically to get EDTA in solution])
  0.5% SDS = 5 ml stock (Stock 10% = 30.00 g Sodium Lauryl Sulfate [Fisher Scientific S529-500] + 300 ml DDH$_2$O)

- 2-Propanol (Fisher Scientific A416-1)

- Chloroform (Fisher Scientific C298-500)

APPENDIX 2
NORTHERN BLOTTING

AGAROSE GEL ELECTROPHORESIS PROTOCOL

DONE

- Preparation of the electrophoresis apparatus:

___ Before transferring the gel, pour 1 X running buffer to the electrophoresis apparatus.

___ Attach the circulation unit in such a way that the buffer runs from (+) to (-) direction.

___ Fill the circulation hose with buffer by circulating the buffer briefly.

- Sample preparation:

___ Aliquot the total RNA (5-30 μg) into 0.5ml bullet tubes.

___ Lyophilize the samples using a Speed Vac for 15-20 minutes.

___ Add 15 μl sample denaturing solution to each tube.

___ Hold on ice for a minimum of 2 hr.

___ Heat in 65 °C water bath for 15 min.

___ Rapidly chill on ice.

___ Add 5 μl loading buffer.

___ Vortex, spin, and load.

- Electrophoresis:

___ Run at 100 volt for 10-15 min until dye migrates into gel.

___ Adjust to 24 volts to run one row-overnight.

___ Turn on the circulation switch (120 volt).

___ 1 hr before the completion of electrophoresis, add 1% EtBr to the buffer (10 μl per liter buffer; 25μl total) and gently swirl.

___ At completion, visualize RNA under UV.

BLOTTING

DONE

___ Fill the reservoir with approximately 500 ml SSC and set up a platform.

___ Cut a wick (3 MM Whatman filter; 4.5 by 8 inches). Soak the wick with transfer solution (20 X SSC). Place the whatman paper to cover the platform. The ends of the whatman paper dip into the 20 X SSC reservoir and serve as a wick.

Smooth the paper over the platform with gloved hand to remove air bubbles that may be trapped.

___ Measure the gel and cut a piece of nylon filter paper (Biotrans Nylon Membranes, 0.2 micron; ICN ESA 2200) to the exact size (4 by 8 inches). Soak the membrane in H_2O for 5 minutes. Pour off the H_2O and soak the membrane in 20 X SSC. Cut two more pieces of whaterman paper of the same size as the nylon membrane and soak them in 20 X SSC.

___ Place the gel on top of the wick. Remove any air bubbles that may be trapped between the membrane and the gel. Place Saran wrap around the edges of the gel so that no part of the membrane can directly contact the whaterman paper wick.

___ Place the presoaked nylon filter on top of gel.

___ Place 2 layers of 20 X SSC-presoaked Whatman filter (3 MM) on top of the nylon filter.

___ Place paper towels (~5 cm thickness) on top of Whatmans.

___ Put a flat hard board/glass on top of the paper towel layer.

___ Put a weight on top (~0.5 kg).

___ Transfer RNA overnight and confirm the transfer under UV.

___ Dry the filter for 30 min at RT and bake at 80 °C for 2 hr.

___ Put the nylon filter in hybridization bag and store at 4°C until used.

REAGENTS AND SOLUTIONS

- 25 X Running Buffer (0.5 M phosphate buffer, pH 7.0):
  1000 ml of stock solution are prepared as follows:
  60 g NaH_2Po_4 monosodium phosphate anhydrous (Fisher Scientific, BP329-500).
  70.98 g Na_2HPo_4 disodium phosphate anhydrous (Fisher Scientific, BP332-500).
  Mix 0.5 M monosodium phosphate and disodium phosphate stock solutions at 39:61 (ml).
  Add 80 ml DDH_2O and adjust pH to 7.0.
  Add dw to 200 ml.
  Autoclave or filter-sterilize through 0.45 micron and store at RT.

- 10 X Denaturing Buffer:
  6.766 g Hepes [4(-2-hydroxyethyl)-1-piperazineethane sulfonic acid] (Fisher Scientific, BP310-100).
  0.680 g sodium acetate trihydrate (Fisher Scientific, BP334-500).
  0.372 g EDTA (Fisher Biotech, BP 120-1000).
  Adjust pH to 7.0 with NaOH and volume to 100 ml.
  Autoclave or filter (0.45 micron), wrap in foil and store at 4 °C.

- Sample Denaturing Buffer (do not store more than 2 wks):
  0.72 ml 10 X denaturing buffer.
  3.0 ml 90% deionized formamide (Fisher Scientific, BP227-500).

1.07 ml 37% w/w deionized formaldehyde, (Fisher Scientific, F79-1).
1.21 ml DDH_2O (final volume=6.00 ml; pH 7.5).
Filter through 0.45 micron. Store in plastic tube wrapped in foil at 4 C.

- Loading Buffer:
  0.4 ml 25 X running buffer.
  5.0 ml glycerol (Fisher Scientific, BP229-1).
  4.6 ml DDH_2O
  5 mg bromophenol blue (Sigma B-6896).
  Filter through 0.45 micron and store at 4 °C wrapped in foil.

- Agarose gel (1.5%):
  8 ml 25 X running buffer.
  158 ml DDH_2O.
  3 g agarose (Fisher Biotech, BP164-100).
  Heat the solution in microwave for 1 to 2 minutes.
  Add 32.1 ml deionized formaldehyde and pour onto the mould (make sure that there are no air bubbles).
  Allow the gel to polymerize for at least 1 hr (2 hr preferred) in the hood.

- 20X SSC:
  701.2 g NaCL (Fisher Scientific BP358-212).
  352.8 g Na Citrate (Fisher Scientific BP327-1).
  DDH_2O to 4 liters.
  pH 7.0.

APPENDIX 3
DOT BLOTTING

SAMPLE PREPARATION

DONE

___ Pipet 2.5, 5, 10 or 20 µg of each RNA sample into sterile bullet tubes. Speed Vac the samples.
___ Dilute RNA with 250 µl of denaturing buffer.
___ Incubate the samples at 65°C water bath for 5 minutes.
___ Add 250 µl of 20X SSC to each tube. Vortex mix and centrifuge briefly for 5 to 10 seconds. The sample is ready to load.

FILTER PREPARATION

DONE

___ Cut a piece of BioTrans nylon membrane (ICM Biomedicals, INC; 0.2 microns; cat 810300) of 10 by 13.3 cm. Soak the membrane in water and then in 10X SSC.
___ Soak the Schleicher & Schuell filter paper (for the dot blot apparatus) in DDH_2O first an in 10X SSC later.
___ Place one layer of filter paper on the blotting unit (Minifold®; Schleicher & Schuell, Inc). Place the BioTrans membrane on top the filter paper.
___ Clamp the blotting unit together.

LOADING OF RNA SAMPLES

DONE

___ Prefilter the wells with 500 µl of 10X SSC. Turn on the vacuum so the buffer moves through the wells slowly.
___ Load the RNA samples and elute slowly. Turn on the vacuum only after all RNA samples have been loaded.
___ Once all the samples have been filtered through, wash each well with 500 µl of 20X SSC.
___ Disassemble the apparatus, mark the filter for the orientation and allow it to air dry.
___ Place the membrane between two pieces of Whatman paper and bake at 80°C for 2 hrs.

REAGENTS AND SOLUTIONS

- Denaturation Buffer (50% formamide, 6% formaldehyde, 20mM Tris, pH 7.0):
  0.242 g Tris (Fisher Scientific BP152-1)
  50 ml Formamide (Fisher Scientific BP227-500)
  6 ml Formaldehyde 37% w/w (Fisher Scientific F79-1)
  DDH$_2$O up to 100 ml. Adjust pH to 7.0

- 20X SSC:
  175.30 g NACL (Fisher Scientific BP358-212)
  88.30 NA Citrate (Fisher Scientific BP327-1)
  DDH$_2$O to 1000 ml, pH 7.0

- BioTrans nylon membrane:
  ICM Biomedicals, INC; 0.2 microns; cat 810300; Aurora OH (10 by 13.3 cm)

- Blotting unit:
  Minifold®; Schleicher & Schuell, Inc.; Keene, NH.

APPENDIX 4
PREHYBRIDIZATION AND HYBRIDIZATION

PROTOCOL

DONE

__ Prehybridize the blot for at least 15 minutes in prehybidization/hybridization solution at 60-65°C (for homologous DNA).

__ **Boil the probe for 5 minutes** and chill rapidly. Add the probe to the hybridization solution at the desired concentration.

__ Add the hybridization solution containing the probe to the blot and hybridize for at least 16 hrs at 60-65°C in the shaking water bath.

__ Prepare the wash solution by heating to 65°C

__ Remove the blot from bag and wash 2 times for 15 minutes each at 60-65°C in washing buffer.

__ Check the blot with a Geiger counter. Wash another 1 or 2 times if necessary.

REAGENTS AND SOLUTIONS

- 1 M Na_2HPo_4 (500 ml):
  70.98 g sodium phosphate dibasic anhydrous (Fisher Biotech, BP332-500).
  450 ml DDH_2O.
  pH with 85% phosphoric acid (Fisher Scientific, A242-212) to pH 7.4.
  Qs to 500 ml with DDH_2O.

- Prehybridization/hybridization solution (100 ml; 0.5 M Na_2HPo_4, 7% SDS, 1% BSA, 10mM EDTA):
  1 g BSA (Sigma A-7888).
  2 ml 0.5 M EDTA (Fisher Biotech, BP 120-1000).
  48 ml DDH_2O (Millique, warmed).
  50 ml 1M Na_2HPo_4 (warmed)
  Mix together and stir until dissolved. Add 7 g of SDS (Fisher Scientific, S529-500). Put solution in water bath at 65°C. The SDS will go into solution fairly quickly.

- Washing solution (1000 ml; 0.1% SDS, 40 mM phosphate):
  7 g of SDS
  40 ml 1M Na_2HPo_4

Qs 1000 ml with DDH_2O
For higher stringency, use 20 mM Na_2HPo_4 and 0.1% SDS.

APPENDIX 5
MULTIPRIME DNA LABELING (AMERSHAM KIT RPN.1601)

DONE

___ Place the required tubes from the Amersham Multiprime DNA labeling system, with the exception of the enzyme (solution 3), in an ice bath to thaw. Leave the enzyme at -20°C until required, and return to the freezer immediately after use.

___ Aliquot 25 ng of cDNA. Denature the sample by heating at **95-100°C** for **2 minutes** in boiling bath water, then chill on ice.

___ To a conical polypropylene reaction tube (500 μl capacity), placed in ice bath, add the appropriate volume of each reagent in the following order:

cDNA probe (5ng/μl)		5 μl
Buffer	Solution No.1	10 μl
Primer	Solution No.2	5 μl
DDH$_2$O		20 μl
Radiolabeled [α-^{32}P]dCTP		8 μl
Enzyme	Solution No.3	2 μl

___ Mix gently by pipetting up and down and cap the tube. Spin for a few seconds in a microfuge to collect the contents in the bottom of the tube. Avoid vigorous mixing because loss of enzyme activity may result.

___ Incubate at 37°C for 30 minutes.

___ Add 50 μg of yeast RNA (0.65 μl of 77 μg/μl of stock).

___ Add 50 μl DDH$_2$O.

___ Add 100 μl phenol (Fisher Scientific).

___ Vortex and spin. Immediately apply on a G-50 gel column made with sterile pipet.

___ Elute with 100 μl sterile DDH$_2$ and collect each fraction in a numbered conical polypropylene tube (500 μl capacity, 25 tubes).

___ Monitor with Guyger counter. Labeled DNA starts eluting at Fraction No.7-8.

APPENDIX 6
POLY-A RNA ISOLATION

PROTOCOL

DONE

___ For each sample, weight out 60 mg of oligo(dt)-cellulose type 7 (Pharmacia 27-5543-02) into a microfuge tube. Add 600 µl of Na-TES, swirl to mix. Centrifuge at 12,000 rpm for 5 minutes at room temperature. Discard supernatant. Repeat twice. Resuspend the pellet in 300 µl NA-TES (final concentration is 100 mg/ml, volume is 600 µl).

___ Add 60 µl of 10X TES and 540 µl of sterile DDH_2O to total RNA sample (final volume 600 µl of 1X TES, in a sterile microfuge 1.5 ml tube).

___ Heat RNA for 5 minutes at 65°C and cold on ice.

___ Add 60 µl of 5M NaCl (1/10 th volume) to RNA sample already suspended in 600 µl of hydrated oligo (dt) cellulose and mix thoroughly at room temperature.

___ Incubate the RNA-oligo (dt) cellulose mixture at 37°C for 10 minutes with occasional gentle mixing by inversion.

___ Centrifuge at 12,000 at room temperature for 5 minutes to pellet the oligo (dt) cellulose and remove the supernatant (fraction A). Keep fraction A (contains total RNA-ply-A RNA) at -80°C until you are sure poly-A has been isolated.

___ Wash the pellet with 1 ml of Na-TES and centrifuge (fraction B).

___ Elute the poly-A RNA by resuspending the pellet in 400 µl of autoclaved DDH_2O and heating at 55°C for 5 minutes and centrifuge (fraction C).

___ Repeat the elution procedure (step 8) twice (fractions D & E).

___ Precipitate fractions C, D & E by adding 1/10th volume of 2M sodium acetate (pH 5.0) and 1 volume of isopropanol. Mix gently but thoroughly. Allow to precipitate overnight at -70°C freezer.

__ Spin down the RNA in a microfuge at 4°C for 45 minutes.

__ Discard supernatant and wash the pellet in 70% ethanol. Recentrifuge for 15 minutes. Discard ethanol and blot the tube dry with a Q-tip. Allow the ethanol to evaporate off for 5-15 minutes. Resuspend the RNA in 30 μl of sterile DDH$_2$O (10 μl/fraction and pool the fractions).

__ Make a 1:100 dilution of the RNA by taking 5 μl of sample and adding 495 μl of sterile DDH$_2$O. Read the OD$_{260}$ of the diluted sample on the spectrophotometer and calculate the yield using the following formula:
OD$_{260}$ X dilution factor (100) X 33 μg/ml X total sample volume = μg RNA. Yields 10-25 μg RNA for 1 mg of total RNA.

SOLUTIONS

- Na-TES: (0.5M NaCl, 10mM Tris-HCL pH 7.5, 1mM EDTA, 0.1% SDS) (*Autoclave, stored at room temperature*). For 50 ml:
  5 ml 5M NaCL + 500μl 1M Tris-HCL (pH 7.5) + 100μl 0.5M EDTA + 500μl 10% SDS + DDH$_2$O to 50 ml.

- TES: (10mM Tris-HCL pH 7.5, 1mM EDTA, 0.1% SDS) (*Autoclave, stored at room temperature*). For 50 ml of 10X solution:
  500 ml 1M Tris-HCL (pH 7.5) + 1 ml 0.5M EDTA + 5 ml 10% SDS + DDH$_2$O to 50 ml.

- 5M NaCl: (for 100 ml, *Autoclave, stored at room temperature*)
  29.22 g NaCl (Fisher Biotech, BP358-212) in 100 ml DDH$_2$O.

- 1M Tris: 88.26 g Tris-Hydrochloride (Fisher Biotech, BP153-1000) and 53.28 g Tris-Base (Fisher Biotech, BP152-1000); add up to 1 liter, adjust pH to 8.0 (*Autoclave, stored at room temperature*)

- 0.5M EDTA: 18.61 g EDTA in 100 ml DD H$_2$O. pH to 8.0 (Fisher Biotech BP120-1000, Molecular Biology Reagents, *Autoclave, stored at room temperature*)

- 2M Sodium Acetate: 27.21 g dissolved in 100 ml DD H$_2$O (Fisher Biotech BP326-500, enzyme grade)(*Autoclave, stored at room temperature*).

APPENDIX 7
IGF-I and IGF-II RIA IN FOLLICULAR FLUID

PREPARATION OF SAMPLES

IGFBPs are removed from each sample using SEP-PAK C_{18} cartridges (Waters, Milford, MA; Part No. 20515)

1. Dispense 1300 μl of 1% aqueous trifluoroacetic acid (TFA) into 12 X 75 mm polystyrene tubes (Sarstedt; Part No. 55.476)

2. Add 200 μl of follicular fluid

3. Stand the mixture at room temperature for 10 minutes

4. Condition of SEP-PAK C_{18} cartridges:
    3 ml acetonitrile,
    3 ml DDH_2O,
    3 ml 0.1 % aqueous TFA

5. Load the acidified follicular fluid in the cartridge carefully

6. Wash the cartridge with 1 ml 0.1% aqueous TFA 3 times (steps 5 and 6 removes IGFBPs)

7. Elute the retentate containing free IGFs with 2 ml 0.1 % TFA in acetonitrile

8. Dry the elute by air stream on a 37-40°C water bath

9. Resuspend de dried eluate with 4 ml RIA buffer at 4°C for 3 hrs (vortex)

10. Spin at 3,000 rpm for 30 minutes

11. Store at 4°C for immediate use or at -80°C for 3 to 6 months

SOLUTIONS

- RIA buffer: (per liter)
  3.6 g sodium monobasic anydrate (Fisher Biotech, BP329)
  0.2 g protamine sulfate (Sigma, P-4505)
  0.2 g sodium azide (Sigma, S-2002)
  3.72 g EDTA (Fisher Biotech, BP-120)
  0.5 ml Tween 20 ()
  pH 7.3 at room temperature (7.5 at 4°C)

- 1% Trifluoroacetic acid (TFA): for 200 samples, final volume = 300 ml
  297 ml DDH_2O + 3 ml TFA (Fisher Chemical A 322)

- 0.1% Trifluoroacetic acid (TFA): for 200 samples, final volume = 1200 ml
  (1198 ml +800) μl DDH_2O + 1.2 ml TFA

- Acetonitrile 0.1% TFA: for 200 samples, final volume = 400 ml
  (399 ml + 600 μl) acetonitrile (Fisher Scientific, A998-4) + 0.4 ml TFA

IGF-I RIA

1. Dispense 400 μl of RIA buffer to NSB and TC, and 300 μl of RIA buffer to BO

2. Dispense 100 μl of standard solutions (range 5-640 pg/tube), and 300 μl of RIA buffer to the standard curve

3. Dispense 20 μl of resuspended eluate (standards, buffer and unknown) to al tubes. Dispense 280 μl of RIA buffer (standards, buffer and unknown) to al tubes

4. Add 100 μl of first antibody (UBK 487; National Hormone and Pituitary Program; rabbit antibody) to all tubes except NSB and TC

5. Add 100 μl of radiolabeled IGF-I (15,000-20,000 cpm) to all tubes

6. Incubate overnight at 4°C

7. Add 100 μl of second antibody (sheep antiserum to rabbit τ-globulin; Dr. Head??) to all tubes. Incubate at 4°C for 1 hr

8. Add 100 μl of normal rabbit serum to all tubes. Incubate at 4°C for 1 hr

Separation:

1. Add 1 ml RIA buffer, and centrifuge at 3,000 rpm for 30 minutes

2. Aspirate the supernatant and count the pellet in a gamma-counter

SOLUTIONS

- IGF-I standard curve:
  To prepare one assay standard curve:
  10 μl (1 ng/1 μl) + 990 μl RIA buffer= 1000 μl (1000 pg/100 μl;1:100)
  A: 1000 pg/100 μl * X = 640 pg/100 μl + 600 μl (final volume)
  X = (640*600)/1000 = 384μl
  B: 384 μl A + 216 μl RIA buffer = 600 μl (640 pg/100 μl)
  C: 250 μl B + 250 μl RIA buffer = 500 μl (320 pg/100 μl)
  D: 250 μl C + 250 μl RIA buffer = 500 μl (160 pg/100 μl)
  E: 250 μl D + 250 μl RIA buffer = 500 μl (80 pg/100 μl)
  F: 250 μl E + 250 μl RIA buffer = 500 μl (40 pg/100 μl)
  G: 250 μl F + 250 μl RIA buffer = 500 μl (20 pg/100 μl)
  H: 250 μl G + 250 μl RIA buffer = 500 μl (10 pg/100 μl)
  I: 250 μl H + 250 μl RIA buffer = 500 μl (5 pg/100 μl)

- IGF-I first antibody: (UBK 487; National Hormone and Pituitary Program; rabbit antibody) final dilution should be 1:20.000 (now is 1:4000)
  To prepare one assay of 200 samples each:
  Stock is already diluted 1:10 and is further diluted 400 fold to have a final dilution of 1:400
  Final volume = 20 ml
  50 μl antiserum + (950 μl + 19 ml) RIA buffer

- IGF-I second antibody: (sheep antiserum to rabbit τ-globulin; Dr. Head??) final dilution should be 1:10 (now is 1:4)
  To prepare one assay of 200 samples each:
  Final volume = 20 ml
  5 ml antiserum + 15 ml RIA buffer

- IGF-I Normal rabbit serum: final dilution should be 1:100
  To prepare one assay of 200 samples each:
  Final volume = 20 ml
  200 μl antiserum + (800 μl + 19 ml) RIA buffer

IGF-II RIA

1. Dispense 400 μl of RIA buffer to NSB and TC, and 300 μ of RIA buffer to BO

2. Dispense 100 μl of standard solutions (range 10-1,280 pg/tube), and 300 μl of RIA buffer to the standard curve

2. Dispense 10 μl of resuspended eluate (standards, buffer and unknown) to al tubes. Dispense 290 μl of RIA buffer (standards, buffer and unknown) to al tubes

3. Add 100 μl of first antibody (mouse monoclonal Ig G to rIGF-II; Amano) to all tubes except NSB and TC

4. Add 100 μl of radiolabeled IGF-II (15,000-20,000 cpm) to all tubes

5. Incubate overnight at 4°C

6. Add 100 μl of second antibody (sheep or goat antimouse IgG (Sigma M-3014) to all tubes. Incubate at 4°C for 1 hr

7. Add 100 μl of normal mouse serum to all tubes. Incubate at 4°C for 1 hr

Separation:

1. Add 1 ml RIA buffer, and centrifuge at 3,000 rpm for 30 minutes

2. Aspirate the supernatant and count the pellet in a gamma-counter

SOLUTIONS

- IGF-II standard curve:
  To prepare one assay standard curve:
  20 μl (1 ng/1 μl) + 980 μl RIA buffer= 1000 μl (2000 pg/100 μl; 1:50)
  A: 2000 pg/100 μl * X = 1280 pg/100 μl + 600 μl (final volume)
  X = (1280*600)/2000 = 384μl
  B: 384 μl A + 216 μl RIA buffer = 600 μl (1280 pg/100 μl)
  C: 250 μl A + 250 μl RIA buffer = 500 μl (640 pg/100 μl)
  D: 250 μl B + 250 μl RIA buffer = 500 μl (320 pg/100 μl)
  E: 250 μl C + 250 μl RIA buffer = 500 μl (160 pg/100 μl)
  F: 250 μl D + 250 μl RIA buffer = 500 μl (80 pg/100 μl)
  G: 250 μl E + 250 μl RIA buffer = 500 μl (40 pg/100 μl)
  H: 250 μl F + 250 μl RIA buffer = 500 μl (20 pg/100 μl)
  I: 250 μl G + 250 μl RIA buffer = 500 μl (10 pg/100 μl)

- IGF-II first antibody: (mouse monoclonal Ig G to rIGF-II; Amano) (0.2 ng) final dilution should be 1:25.000
  To prepare one assay of 200 samples each:
  Dilute 1:10 and then use 2μl every 5 ml
  Final volume = 20 ml
  8 μl antiserum + (992 μl + 19 ml) RIA buffer

- IGF-II second antibody: sheep or goat antimouse IgG (Sigma M-3014) final dilution should be 1:10
  To prepare one assay of 200 samples each:
  Final volume = 20 ml
  2 ml antiserum + 18 ml RIA buffer

- IGF-II Normal mouse serum: final dilution should be 1:100
  To prepare one assay of 200 samples each:
  Final volume = 20 ml
  200 µl antiserum + (800 µl + 19 ml) RIA buffer

APPENDIX 8
LIGAND BLOTTING FOR IGF-BINDING PROTEINS

ONE DIMENTIONAL GEL ELECTROPHORESIS
(Laemmli Gel, Roberts Procedure)

PROCEDURE

DONE

__ 1. Glass Plates Preparation:

Soak in concentrated nitric acid overnight (if not available, use 1.0 % SDS)
Rinse in DDH_2O followed by ethanol
Dry in air

__ 2. Fabricate the Gel Mold:

__ 3. Preparation of Gel:

Mix the gel ingredients according to the recipe listed below (2 gels)

Solution	7.5%	10.0%	12.5%	15.0%	SG^a
Acrylamide 40 % ^{b}(ml)	21.09	28.13	35.16	42.20	3.60
2.5% Bis Acrylamide (ml)	9.00	12.00	15.00	18.00	1.54
Running Buffer (ml) 28.13	28.13	28.13	28.13	4.00^c	
20% SDS (ml)	0.57	0.57	0.57	0.57	0.16
TEMED (μl)	8.25	8.25	8.25	8.25	10.20
DDH_2O	31.20	21.16	11.13	1.09	12.26
Ammonium Persulfate ^{d} (ml)	22.50	22.50	22.50	22.50	10.60

[a] Stacking Gel(3.0%)

[b] 40% Acrylamide (Fisher Biotech, BP1402-1)

[c] Stacking Gel Buffer

[d] **Should be added last**

__ 4. After adding ammonium persulfate to the mixture of gel ingredients, shake several times and pour into the mold using a 60 ml syringe and narrow tubing up to 1 cm bellow the bottom of the well comb

__ 5. Apply several drops of 0.1% SDS on top of the gel

__ 6. After solidification (1 hr), remove 0.1% SDS, pour the stacking gel mixture, and insert the well comb

__ 7. When ready, remove the well comb and wash the wells with chamber buffer a few times

__ 8. Attach the gel fixture to the electrophoresis apparatus, pour chamber buffer and watch for any leakage

__ 9. Loading:
Prepare samples in sample buffer ([final]=1X-2X)
For reducing gel, add 5-10% BME to the sample
Make a hole with a needle in the cap of the bullet tube
Boil 3-5 minutes
Load the samples and the MW markers
Transfer the gel fixture to the electrophoresis chamber
Remove air bubbles at the bottom of the gel by using a glass spoid

__ 10. Electrophoresis:
Run at 25 mV and 25 mA/gel until dye front reaches within 1 cm from the bottom (6-7 hrs).

REAGENTS AND SOLUTIONS

- Running Gel Buffer (1.5 M Tris):
  42.25 g Tris Base (Fisher Biotech, BP152-1000)
  175 ml DDH$_2$O
  0.8 ml TEMED (N,N,N,N'-Tetramethylethylenediamine; BioRad 161-0801)
  Titrate to pH 8.8 with HCL
  Adjust to 250 ml
  Filter (Nalgene size:0.45 mic), degas, and store in brown bottle at 4°C

- Stacking Gel Buffer:
  14.95 g Tris Base
  100 ml DDH$_2$O
  1.15 ml TEMED
  Titrate to pH 6.8 with HCL
  Adjust to 250 ml
  Filter, degas, and store in brown bottle at 4°C

- Acrylamide Bis (2.5%):
  10 g Bis Acrylamide (Fisher Biotech, BP171-100)
  400 ml DDH$_2$O
  Filter, degas, and store in brown bottle at 4°C

- Chamber Buffer:
  12.00 g Tris Base

57.60 g Glycine (Fisher Biotech, BP381-1)
4.00 g Sodium Dodecyl Sulfate (SDS, Hoeffer Scientific, B44244-4H) or Sodium Lauryl Sulfate (SLS, Fisher Scientific, S529-500)
4000 ml DDH$_2$O

- Ammonium Persulfate:
  0.28 g Ammonium Persulfate (Fisher Biotech, BP179)
  100 ml DDH$_2$O
  Prepare on the day to be used

- 20 % SDS:
  10.00 g SDS or SLS
  50 ml DDH$_2$O
  Store at room temperature

- Sample Buffer (2X):
  1.51 g Tris Base
  12.5 ml DDH$_2$O
  Titrate to pH 6.8 with HCL
  10.00 g SDS or SLS
  15 ml Glycerol (Fisher Scientific,00-000) **or** 20 g Sucrose (Fisher Scientific, S5-500)
  Adjust to 100 ml
  Add a few drops of Bromophenol Blue (1g.ml, 0.029 % W/V; Sigma, B-6896)

- Commasie Brilliant Blue Staining Solution:
  1.5 g Commasie Brilliant Blue (CBB, ?)
  750 ml Methanol (Fisher Scientific, A412-4)
  Dissolve the CBB in methanol overnight
  645 ml DDH$_2$O
  105 ml Acetic Acid (Glacial, Fisher Scientific, A38^{s}212)

- Fixing Solution:
  400 ml Ethanol
  70 ml Acetic Acid
  530 ml DDH$_2$O

- Destining Solution:
  1575 ml DDH$_2$O
  135 ml Acetic Acid
  90 ml Methanol

ELECTROTRANSFER

DONE

__ 1. Before stopping SDS-PAGE, soak 2 pieces 3MM Whatman paper and a piece of nitrocellulose filter (0.2-0.45 micron) in Towbin buffer

__ 2. Make a sandwich (-) **sponge-Whatman filter-gel-nitrocellulose filter-Whatman filter-sponge (+)**

Never let the gel and filters run dry
Remove air bubbles between layers of the sandwich
The nitrocellulose filter is directed to the (+) electrode

__ 3. Transfer the proteins either at:

3 hrs, 40 volts, 4°C
3 hrs, 30 volts, RET
Overnight, 200 mA, 4°C

__ 4. After transfer, mark the well position on the nitrocellulose filter

__ 5. Dry the filter for 30 minutes at RT or 5 minutes at 37 °C oven

__ 6. Cut the lane for molecular markers, stain with fast green or amidoblock (Bio-Rad). Destain in 50% DDH_2O-40% Methanol-10% acetic acid

BLOTTING

__ 7. Rinse the dried filter with TBS on a rocker platform 3 times (100 ml TBS, 5 minutes each wash)

__ 8. Block the filter with 100 ml of 1% Blotto on a rocker platform for 1 hr at RT

__ 9. Wash the filter for 10 minutes with TBS-0.1% Tween 20

__ 10. Incubate the filter with ^{125}I IGF-I or IGF-II (preferred, 500.000 cpm/5ml) overnight at 4 °C in a plastic bag on a rocker platform with very low shaking (3 hrs at RT)

Incubation: in TBS-0.1% Tween 20-1% BSA, 5ml/100 cm^2 membrane
Input radioactivity: 0.5-1.0 x 10^6 cpm/5ml

__ 11. After incubation, wash the filter with TBS out a squirt bottle

__ 12. Wash the filter with TBS-0.1%Teen 20 two times, 15 minutes/wash , on a rocker platform

__ 13. Wash with TBS two or three times, 15 minutes/wash, on a rocker until the background drops to <1000 cpm scanned with the Geiger counter

__ 14. Dry at RT and expose to X-Ray film at -70 °C

REAGENTS

- Towbin buffer (transfer buffer):
  15.92 g Tris Base (Fisher Biotech, BP152-1000)
  76.08 g Glycine (Fisher Biotech, BP381-1)
  4200 ml DDH_2O
  1050 ml Methanol (Fisher Scientific, A412-4)

Titrate to pH 8.3
Degas and store at 4°C until use

- Tris Buffer Saline (TBS):
  3.03 g Tris Base
  21.92 g Sodium Chloride (Fisher Scientific, BP358-212)
  1.25 g sodium azide (Sigma, S-2002)
  2500 DDH_2O
  Titrate to pH 7.4 with HCL

- TBS-0.1% Tween 20:
  799.2 ml TBS
  0.8 ml Tween 20 (Fisher Scientific, BP337-100)

- 1% Blotto:
  5 g nonfat dry milk
  500 ml TBS

- TBS-0.1% Tween 20 1%-BSA:
  0.2 g BSA
  20 ml TBS
  20 μl Tween 20 (0.1%, Fisher Scientific, BP337-100)

APPENDIX 9
IMMUNOPRECIPITATION OF IGF-BINDING PROTEINS

EXPANSION OF PROTEIN A-SEPHAROSE

DONE

__ 1. Expand 0.5 g of Protein A-Sepharose CL-4B (Pharmacia, Piscataway, NJ) in 50 ml of PBS over rocking platform at RT for 4 hrs,
__ 2. Centrifuge at 3,000 rpm for 10 min,
__ 3. Wash twice with 50 mM Tris (pH 7.4),
__ 4. Centrifuge at 3,000 rpm for 10 min,
__ 5. Dilute 50% in 50 mM Tris, (pH 7.4). Store at 4°C.

IMMUNOPRECIPITATION

DONE

__ 1. Mix 250 μg of protein and distilled water (final volume=25 μl; positive control),
__ 2. Mix 250 μg of protein and distilled water (final volume=20 μl) + 5 μl of IGFBP antiserum (2, 3, 4, 5 and 6; samples),
__ 3. Mix 250 μg of protein and distilled water (final volume=20 μl) + 5 μl of normal rabbit serum (negative control),
__ 3. Add the mixture to 250 μl washed μl of Protein A-Sepharose CL-4B along with 500 μl 50mM Tris,
__ 5. Incubate overnight on a rotating shelf at 4°C
__ 6. Microfuge for 1 min and save the pellet,
__ 7. Add 500 μl of TT, vortex, microfuge 1 min and discard supernatant. Repeat 3 times,
__ 8. Solubilize al samples in 75 μl of 2X sample buffer (final 1X),
__ 9. Boil for 3 min, microfuge down the Asepharose and load the top aqueous layer,
__10. Follow one dimensional gel (Laemmli, 1970) and ligand blot (Lee *et al.*, 1991) protocols,
__11. Load one lane with pool with no IGFBPs antiserum (positive control), one lane with each pool and corresponding antiserum (IGFBP-2, 3, 4, 5 and 6), and one lane with pool and normal rabbit serum (negative control).

REAGENTS AND SOLUTIONS

- 50 mM Tris (pH 7.4; 300 ml): 15 ml 1M Tris + 285 ml DDH$_2$O.

- 50 mM Tris, 0.5% Trion X-100 (TT; 100 ml): 99.5 ml 50 mM Tris + 0.5 ml Triton X-100.
- 50 mM Sodium Phosphate (pH 7.4, 100 ml): 5 ml 1M $NaPo_4$ + 95 ml DDH_2O.

BIOGRAPHICAL SKETCH

Rodolfo Luzbel de la Sota (h), the only son of Elsa Luisa Pagliari and Rodolfo Luzbel de la Sota, was born in Mercedes, Buenos Aires, on October 8, 1959. He attended elementary school in Mercedes, and secondary school in Buenos Aires and in Carlos Casares. He was accepted as a student to the Faculty of Veterinary Sciences of the National University of La Plata in March of 1978 where he received his degree of Veterinary Surgeon on March 22nd, 1983. In July of 1983, he was awarded a fellowship from the National Council of Scientific Research from Argentina for a three year period to do research in the area of bovine mastitis control. In January of 1987, he came to the University of Florida sponsored by a scholarship from the Rotary Foundation of Rotary International to pursue his degree of Master of Science under the supervision of Dr. Roger Natzke which he obtained in January of 1990. The main focus of this thesis was the study of clinical mastitis from an epidemiological perspective and development of economic indices utilizing DHIA data from Florida and Georgia dairy herds.

In May, 1990, he began his doctoral program under the direction of Dr. William W. Thatcher in the Department of Animal Science at the University of Florida. After completion of the degree of Doctor of Philosophy, he will return to Argentina where he will work at the National Institute for Research in Animal and

Agricultural Technology in Rafaela, Santa Fe and at the Department of Theriogenology and Animal Production Medicine at the Faculty of Veterinary Sciences of Esperanza, Sante Fe. He is married to Teresita Miquelarena and has two sons. Tomás was born on February 18, 1989, and Matías was born on January 5,1992.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William W. Thatcher

William W. Thatcher, Chairman
Graduate Research Professor
of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Frank

Frank A. Simmen
Associate Professor of Dairy and Poultry Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maarten Drost
Professor of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter J. Hansen
Professor of Dairy and Poultry Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lynn H. Larkin

Lynn H. Larkin
Professor of Anatomy and Cell Biology

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May, 1995

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School